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THE BACTERICIDAL PROPERTIES OF THE QUATERNARY SALTS OF HEXAMETHYLENETETRAMINE.

I. The Problem of the Chemotherapy of Experimental Bacterial Infections.

By WALTER A. JACOBS, Ph.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, March 1, 1916.)

The researches of Ehrlich and his coworkers during the past decade, which led to the discovery of salvarsan, have created a new procedure in the search for remedies for the control of infectious diseases. The discovery of this drug was the result of a logically conducted development of the chemistry of organic arsenic compounds in the directions indicated by their biological properties. This new procedure may be regarded in a way as a revival of the one in use during the time preceding the period of purely biological method, for at that time the therapeutic values of quinine, mercury, arsenic, and salicylic acid were first discovered. The resemblance of the old to the new consists essentially in the fact that in each case chemical substances were used as the therapeutic agents, with the difference, however, that the finding of the old chemical remedies in no way involved the use of synthetic organic chemistry, controlled by constant biological cooperation, the essential feature of the new chemotherapeutic method. This new departure must not be confused with the older synthetic pharmacology, the aim of which was quite different.

A few workers, and the number is constantly increasing, have been attracted to the new field, and the occasional report of promising results, taken with the drugs already proven of therapeutic value, affords ample justification for greater expectations. But to each worker the first and perhaps greatest problem which presents itself is the finding of leads. Salvarsan, optochin, and the trypan dyes, the chief products of the new chemotherapy, all owe their discovery to the development of leads which had been furnished by clinical observations in the

field. Arsenic, mercury, quinine, and certain dyes are perhaps the only substances which up to now have been regarded with assurance as leads for chemotherapeutic development. And of these, with the exception of optochin and perhaps of salicylic acid, all have been indicated for protozoan infections only, and there is promise that by the proper development of these substances the problem of the control of most protozoan infections will be successfully solved.

When we turn, however, to the cure of infections of purely bacterial origin the problem which confronts us is found to be more difficult of attack. Here not only the difficulty of procuring a suitable experimental infection as a test object, but the absence of clinical observations which might be regarded in the nature of leads which could form the basis for a rational chemotherapeutic procedure, makes it imperative from the start to seek for such leads. The successful chemical development of quinine which led to the discovery of ethylhydrocuprein already indicates that resource may be had eventually to the above mentioned protozoan leads. And it is a fact that perhaps with the exception of salicylic acid this drug is the first substance to have been used successfully in an experimental bacterial infection. This one fact creates the hope that chemotherapy may find a wider application in the control of bacterial infections. It is conceivable, however, that ultimate success may depend upon the finding of leads other than those which have been successfully used against protozoan infections.

It would seem that a wealth of material should lie ready among the numerous classes of organic substances which have been found to exert powerful bactericidal effects *in vitro*. But the failure of many of these when tried in experimental infections has led to the realization that besides a bacteria-killing property the fulfillment of certain other requirements is essential for the achievement of an internal antisepsis. Some workers believe that no indication of the probable effect of a substance *in vivo* can be discerned from its action *in vitro*, and that the successful control of an infection by chemical agents can be attained only by indirect means. This might occur, on the one hand, as perhaps in the case of atoxyl, by the chemical transformation *in vivo* of the injected drug into some active form. On the other hand, the substance might act through the protective mechanism of

the host either by increasing phagocytosis or by stimulating the production of immunity principles. It is, of course, probable that in some instances these phenomena might play a part and that a possible scheme of chemotherapeutic attack could be developed along these lines.

Owing to the ease of handling bacteria *in vitro* and the simplicity of the bactericidal tests it would be unwise because of former failures to condemn the *in vitro* method, at least as a means of initial orientation. If it were accompanied by certain parallel studies the *in vitro* method should do more than afford only orientating data. As pointed out by Bechhold and Ehrlich[1] and others in the past, besides a mere bactericidal power other conditions must be fulfilled before a substance may be considered even a possibility as a therapeutic agent, provided of course a direct action by the drug itself is in question.

Aside from the obvious conditions of solubility and relative non-toxicity, the drug once in the circulation, whether by direct intravenous injection or by absorption, must be maintained therein for a sufficient length of time and in sufficient concentration in order to unfold its *in vitro* effect. In other words, its free access to the foci of infection should not be completely obstructed.

To accomplish this it must not enter too rapidly into chemical or physical combination with the constituents of the tissues, of which the blood is a fair representative. It must not be too speedily eliminated. And, finally, it must not be too rapidly altered in any way by metabolic processes which would nullify its bactericidal character. There may be still other and less definite factors which separate the *in vitro* from the desired *in vivo* result. If, however, the *in vitro* bactericidal tests could be complemented by a parallel study of those properties of substances which would decide whether they could satisfy the above requirements *in vivo*, our choice of substances for the *in vivo* experiments could be in great measure controlled. A system, though somewhat arbitrary, would be substituted in an undertaking which would otherwise be directed by a haphazard and entirely opportunistic policy.

These considerations have convinced us that the procedure in the search for leads in the chemotherapy of bacterial infections may be

[1] Bechhold, H., and Ehrlich, P., *Ztschr. f. physiol. Chem.*, 1906, xlvii, 173.

logically systematized as follows: Substances which either by their general structure or by the possession of characteristic atomic groups are representative of as many types of organic substances as possible should be systematically selected for bacteriological and biological testing. Such facts as the bactericidal power and partial specificity for certain types, compatibility with tissue constituents (serum), and resistance to profound and rapid metabolic alteration should be noted and considered in the final interpretation of what in the chemical constitution of the substances is responsible for the observed biological behavior.

With organic substances there will be considerable difficulty in satisfying the last requirement. In the case of arsenicals and mercurials it is immaterial whether metabolization should occur, for the therapeutic characteristics of such compounds are elements. Their value may partly depend upon such metabolization. It does not seem improbable, however, that bactericidal substances may be found which, even though to a less degree than the arsenicals and mercurials, may be sufficiently resistant to metabolic changes to enable them to produce a sterilizing effect before they are disposed of by the host. The large number of pharmacologically active preparations must all persist long enough after administration to produce their physiological actions.

From the representative substances which have been found to possess the required biological properties, two classes of leads might be obtained: first, those substances which, like quinine, owe their bactericidal action to the general structure of the molecule; and, second, those which, like phenol, are bactericidal principally because of the possession of a certain atomic group. Once in the possession of *bactericidogenic*,[2] tissue-compatible molecules or side-chains, the same systematic development so successfully employed in the development of organic arsenicals by the alteration or addition of groups to the molecule might be here repeated in order to augment the specific bactericidal action, to detoxify it, or in some other way

[2] The word *bactericidogenic*, of obvious derivation, is employed in this and the following articles as a convenient term to express the property of certain chemical groups, when introduced into an organic molecule, of imparting bactericidal properties to that molecule.

furnish it with biologically desirable properties. In this way substances could be obtained which would form a rational basis for chemotherapeutic investigations.

The problem of the chemotherapy of bacterial infections and a possible scheme for its systematic attack have been discussed above in some detail with the purpose of affording a basis for a better understanding of the material which will be presented in the following papers.

From its nature this material will touch on but one phase of the above scheme and no claim is made of its complete realization. We shall present the results obtained in a systematic attempt to alter chemically the molecule of hexamethylenetetramine with the object of obtaining a class of bactericidal substances which could be employed in experimental infections. The use of this drug was inspired by the interest felt by Dr. Flexner in the possible application of some of its derivatives in the treatment of experimental poliomyelitis, and the material which will here be presented is but a part of a larger undertaking executed with it.

We shall attempt to show how, by the selection of a certain molecular group, namely hexamethylenetetramine, it has been possible to demonstrate its general bactericidogenic character. By the combination of this substance in the form of quaternary salts, in the manner to be described later, with a great variety of other molecular groupings a new class of bactericidal substances has been prepared[3] in which the bactericidal nature was principally attributable to the hexamethylenetetramine nucleus. On the other hand, the degree of this action was determined by the nature of the molecular groups added to hexamethylenetetramine. These added groups were likewise responsible for the partial specificity of certain of the preparations for particular bacterial species. This partial specificity did not favor one species alone, but all the species tested were found to be separately and specifically susceptible to some particular type of hexamethylenetetramine derivative. We must therefore conclude that the bactericidogenic character of hexamethylenetetramine ex-

[3] For the chemistry of these substances and the references to those prepared by others see Jacobs, W. A., and Heidelberger, M., *Jour. Biol. Chem.*, 1915, xx, 659, 685; 1915, xxi, 103, 145, 403, 439, 455, 465.

hibited in its quaternary salts is not specific but general in character. The specificity, however, is furnished by the proper choice of the molecular grouping added.

It will also be shown that a few of the hexamethylenetetraminium compounds which were tested were either not at all or but slightly inhibited by serum. A few, on the other hand, were found to be greatly inhibited by serum. The fact, however, that any one of the hexamethylenetetraminium salts is compatible with serum is enough to demonstrate the serum compatibility of the bactericidogenic hexamethylenetetramine portion of the molecule itself. We have here, therefore, a bactericidogenic, serum-compatible group. The remainder of the molecule determines the serum incompatibility of those substances the action of which was found to be inhibited by serum.

In the same way the toxicity relationships were found to be determined by the groups contained in that portion of the molecule added to the hexamethylenetetramine nucleus.

We can regard the material here presented merely as a beginning, but we feel that such a treatment of the problem as here presented may ultimately result in an accumulation of data which will be of value in the systematic search for substances which may be used in the control of experimental bacterial infections. Before passing judgment, however, on the chances offered by the further development of the quaternary salts of hexamethylenetetramine, the behavior of these substances in the animal organism should be studied in order to determine whether the bactericidogenic group in itself is sufficiently resistant towards metabolic changes. Otherwise these compounds as a class would be bactericidally inert *in vivo*.

THE BACTERICIDAL PROPERTIES OF THE QUATERNARY SALTS OF HEXAMETHYLENETETRAMINE.

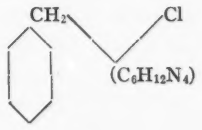
II. The Relation between Constitution and Bactericidal Action in the Substituted Benzylhexamethylenetetraminium Salts.

By WALTER A. JACOBS, Ph.D., MICHAEL HEIDELBERGER, Ph.D., and HAROLD L. AMOSS, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, March 1, 1916.)

Hexamethylenetetramine, as a tertiary nitrogen compound, reacts readily with benzyl chloride, bromide, or iodide, and their numerous nucleus substituted derivatives to form quaternary salts. The results obtained in the study of the bactericidal properties of such substances are the subject of the present communication. In these compounds, the structure of which may be represented as follows,



it is seen that by means of a CH_2 side-chain the hexamethylenetetramine molecule is linked to a benzene nucleus. By the use of a great variety of substituted benzyl halides it was found possible to prepare for study a variety of hexamethylenetetraminium salts[1] in which the benzene nucleus was varied at will in the character, number, and position of the different atoms and groups introduced. By this procedure the opportunity was afforded of studying the effect of chemical constitution upon bactericidal action in a uniform series of substances.

Because the number of substances involved in the investigations

[1] For the chemistry of these substances and the references to those prepared by others see Jacobs, W. A., and Heidelberger, M., *Jour. Biol. Chem.*, 1915, xx, 659; 1915, xxi, 465.

described in these papers was so large, it was found necessary for the practical execution of the bactericidal tests to adopt a scheme which would involve the least amount of work and still furnish a satisfactory measure of the activity of the substances. For this reason the drug dilutions, which as a rule started with 1: 200, were doubled in each successive dilution so that the series 1: 200, 1: 400, 1: 800, 1: 1,600, etc., were the concentrations with which the observations were made. With this scheme it is seen that as the dilutions increase the differences between them become greater, making it possible to regard the figures obtained only as rough approximations to the true values. In the strictest sense account should be taken of the molecular weights of the substances in a direct comparison of their bactericidal properties, but with the dilution scheme here employed this was deemed unnecessary.

In spite of the crudity of our figures it will be seen that certain relationships between the constitution and the bactericidal action are plainly in evidence. The results given below clearly demonstrate that by the addition of hexamethylenetetramine to benzyl chloride a bactericidal substance is obtained, and that by the substitution in the benzene nucleus of different atoms and groups this action may be altered at will, the resulting effect depending upon the number, character, and position of these substituents. In this class of compounds we possess a new group of bactericidal substances in which the hexamethylenetetramine nucleus is directly responsible for their bactericidal character.

EXPERIMENTAL PART.[2]

Technique.—A strain of *Bacillus typhosus* which had been growing on artificial media for several years and which is a good agglutinator was used in testing the germicidal effects of the compounds.

0.5 or 1 per cent solutions of the substance to be tested were made up in physiological salt solution and filtered immediately through a Berkefeld N filter. With sterile salt solution the dilutions of 1: 200, 1: 400, 1: 800, 1: 1,600, 1: 3,200, 1: 6,400, and 1: 12,800 were made and

[2] Some of the tests with *B. typhosus* were performed by Dr. Paul F. Clark, now of the University of Wisconsin, to whom we are greatly indebted for furnishing us with the results of his experiments.

all brought to a temperature of 37°C. To 4 cc. of each dilution, there was added 0.5 cc. of a 24 hour broth culture of *Bacillus typhosus*, and the tubes were put into an incubator or water bath at 37°C. for 4 hours. At the end of this time, one small loopful was taken from each tube and plated in plain agar. It was found necessary to incubate the plates for 48 hours before counting, because some of the colonies did not grow out in 24 hours. Control plates under the same conditions usually contained about 1,000 colonies. Duplicates were run in each case.

Table I presents the bactericidal results obtained with the different preparations tested upon *Bacillus typhosus* by the technique described above. As stated in the introduction, the number of preparations tested and the pressure of other work rendered impossible a detailed study of each substance employed in the tests, so that the figures here given can be regarded only as approximations to the true bactericidal powers of the substances in question. In most instances the figures as given are, if anything, too low. A consideration of the scheme of dilutions employed will show how great the underestimation of the true bactericidal power might be. For example, in the case of the *o*-nitrobenzylhexamethylenetetraminium chloride, given in Table I, the greatest dilution in which this substance killed all the bacteria in 4 hours was 1: 3,200. The next higher dilution tried was 1: 6,400 and this was found ineffective. But if this compound could really kill in a dilution of 1: 5,000 or even 1: 6,000, the value obtained as a result of the scheme of dilution used would be only 1: 3,200. For this reason we must regard the values given only as relative. In spite of this, the alteration in character or position of the substituents in the nucleus was accompanied by changes in the bactericidal action which were too marked to be masked by the dilution scheme employed.

The tests with hexamethylenetetramine itself and the simple aliphatic quaternary salt methylhexamethylenetetraminium iodide showed them to be devoid of action in a dilution of 1: 200. By the substitution in the latter compound of the methyl by the benzyl group the customary influence of the aromatic nucleus was observed. Although not a strong bactericide, the benzyl salt was found to kill all the bacteria present in a dilution of 1: 200. This bactericidal

TABLE I.

Substance.	Killed *B. typhosus* in 4 hrs. at 37°C. in a dilution of 1:
Hexamethylenetetramine	+*
Methylhexamethylenetetraminium iodide	+
Benzylhexamethylenetetraminium chloride	200
o-methylbenzylhexamethylenetetraminium chloride	3,200
m- " "	800
p- " "	800
3, 5-dimethylbenzylhexamethylenetetraminium chloride	400
o-chlorobenzylhexamethylenetetraminium "	1,600
p- " "	800
o-bromobenzylhexamethylenetetraminium "	1,600
p- " "	200
o-iodobenzylhexamethylenetetraminium bromide	1,600
p- " "	1,600
o-cyanobenzylhexamethylenetetraminium chloride	3,200
p- " "	400
o-nitrobenzylhexamethylenetetraminium "	3,200
m- " "	400
p- " "	1,600
2, 4-dinitrobenzylhexamethylenetetraminium "	3,200
o-methoxybenzylhexamethylenetetraminium "	+
p- " "	200
2, 3-dimethoxybenzylhexamethylenetetraminium chloride	+
3, 4- " "	200
3, 4-methylenedioxybenzylhexamethylenetetraminium chloride	200
5-nitro-2-methoxybenzylhexamethylenetetraminium "	400
3-nitro-4- " "	800
2-nitro-3, 4-dimethoxybenzylhexamethylenetetraminium "	3,200
2-acetoxy-3, 5-dibromobenzylhexamethylenetetraminium bromide	1,600
4-acetoxy-3, 5- " "	1,600
2-acetoxy-3, 5-dimethyl-4, 6-dibromobenzylhexamethylenetetraminium bromide	1,600
2-acetoxy-3, 5-dimethylbenzylhexamethylenetetraminium chloride	+
3-carboxy-4-oxybenzylhexamethylenetetraminium "	1,600
3-carbomethoxy-4-oxybenzylhexamethylenetetraminium "	400
2-methoxy-5-carboxybenzylhexamethylenetetraminium "	400
2-methoxy-5-carbomethoxybenzylhexamethylenetetraminium "	400
o-acetaminobenzylhexamethylenetetraminium chloride	800
p- " "	+
1, 2-xylylenedihexamethylenetetraminium dichloride	12,800
1, 3- " "	6,400
Mesitylylenedihexamethylenetetraminium "	12,800

* + indicates growth after exposure to a dilution of 1: 200.

power was further developed by the introduction into the nucleus of various atoms and groups, resulting in the series of substances given in Table I. A study of these brings out the following relationships.

The methyl, chlorine, bromine, iodine, cyano, and nitro groups were all found to increase the bactericidal power of the parent unsubstituted benzyl compound. This behavior of the alkyl, halogen, and nitro group has been frequently observed with other types of organic bactericides; for instance, in the case of the phenols. However, this effect may by no means be regarded as inevitable, as there are many bactericidal substances the power of which is in no way influenced by the introduction of these groups. Examples of this will be found among other types of hexamethylenetetraminium salts to be described in the following paper.

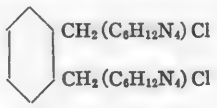
A perusal of the table will show the interesting influence of the position of the substituent upon the bactericidal action. In most cases the *ortho* substituents were found to be more active than either the *meta* or *para* compounds. The regularity of this rule, at least as far as the present series was extended, is striking. The *ortho* methyl, chloro, bromo, nitro, and cyanobenzyl salts were all more active than their other position-isomers.

With this type of hexamethylenetetraminium salt the methoxy group is, on the whole, without influence on the bactericidal effect, if indeed not detrimental. In those compounds which contain the nitro group besides the methoxy group the influence of the former appears to dominate. The 2-nitro-3,4-dimethoxybenzyl derivative was found to equal in effectiveness the 2-nitrobenzylhexamethylenetetraminium chloride. In the same way the 3-nitro-4-methoxy compound was bactericidally about as powerful as the 3-nitrobenzyl salt.

The optimum effect in varying substituents was apparently obtained with the mono- substituted benzyl compounds. In the few cases in which the dimethyl, dibromo, and dinitro derivatives were made, it was found that no advantage as regards the bactericidal value was to be gained, as a rule, by the multiplication of groups. At the same time the greater the number of substituents, particularly in the case of the nitro and halogen compounds, the less was the solubility in water.

In one direction, however, at least when *Bacillus typhosus* was used

as the test object, it was found that a distinct advantage was to be gained by the multiplication of groups; namely, in those compounds in which hexamethylenetetramine was introduced twice into the side-chains. Such salts were obtained by the addition of two molecules of hexamethylenetetramine to ω_1,ω_2-dichloro-*o*-xylene, ω_1,ω_3-dichloro-*m*-xylene, and ω_1,ω_3-dichloromesitylene. These substances possess the following structural formula, in which, of course, the relative positions of the side-chains are different in each case.



These compounds are the strongest bactericides of this group.

Here it should be emphasized that the bactericidal results obtained with this group of substances refer only to their behavior towards *Bacillus typhosus*. As the work developed, other species of bacteria were made the object of an occasional test, but owing to the incompleteness of the results obtained, and to the fact that the technique was varied, it has not been deemed advisable to enlarge on these results in the present paper. It may, however, be said that in general this class of substances was considerably less effective against the streptococcus and meningococcus, but that the results with the gonococcus approached those obtained with *Bacillus typhosus*. This particular group of hexamethylenetetraminium salts cannot, therefore, be regarded as general disinfectants. As a matter of fact, there are but few, if any, organic bactericides which act uniformly against all species or strains of bacteria. A few cases have been selected in Table II to afford a comparison of the effects of several of these salts upon different microorganisms. With the streptococcus, meningococcus, and gonococcus the technique was altered, the time of exposure of the bacteria to the drug being changed to 3 hours and the temperature to 20°. Such a change in technique should, of course, alter the results, but our experience has shown that this rarely exceeded the space of one whole dilution. It is seen from the table that the nature of the substance used determined the effect upon a particular microorganism. A constant relation between the resistances of the various types of organisms is out of the question. The far greater effectiveness

of the two dihexamethylenetetraminium salts against *Bacillus typhosus* is striking. These substances may be classed as "partially specific" for this species.

TABLE II.

Substance.	Killed *B. typhosus* at 37°C. in 4 hrs. in dilution of 1:	Killed streptococcus at 20°C. in 3 hrs. in dilution of 1:	Killed meningococcus at 20°C. in 3 hrs. in dilution of 1:	Killed gonococcus at 20°C. in 3 hrs. in dilution of 1:
Benzylhexamethylenetetraminium chloride	200	+	400	800
p-methylbenzylhexamethylenetetraminium chloride	800	+		
o-bromobenzylhexamethylenetetraminium chloride	1,600	+	+	1,600–3,200
o-cyanobenzylhexamethylenetetraminium chloride	3,200		400	1,600
p-cyanobenzylhexamethylenetetraminium chloride	400		400	800
o-methoxybenzylhexamethylenetetraminium chloride	+*	+	200	800
3, 4-methylenedioxybenzylhexamethylenetetraminium chloride	200	+	1,600	800
o-nitrobenzylhexamethylenetetraminium chloride	3,200	3,200	800	800
m-nitrobenzylhexamethylenetetraminium chloride	400		1,600	1,600
2-acetoxy-3, 5-dibromobenzylhexamethylenetetraminium bromide	1,600	3,200	800	800
2-acetoxy-3, 5-dimethylbenzylhexamethylenetetraminium chloride	+	1,600	800	800
m-xylylenedihexamethylenetetraminium dichloride	6,400	200	+	1,600
Mesitylylenedihexamethylenetetraminium dichloride	12,800	+	400	400

* + indicates growth after exposure to a dilution of 1: 200.

From the consideration of the above observations we feel justified in attributing essentially to the hexamethylenetetraminium group the property of determining the bactericidal character of this class of compounds. For direct comparison with another basic side-chain *p*-nitrobenzylpyridinium chloride was prepared. This was found to be ineffective towards *Bacillus typhosus* even in a concentration of 1: 200

after 4 hours' contact. The corresponding hexamethylenetetramine quaternary salt killed *Bacillus typhosus* in a dilution of 1: 1,600 in 4 hours.

The function, however, of determining the extent and character of this bactericidal property must be attributed to the substituting groups and to the positions occupied by them in the benzene nucleus to which the hexamethylenetetramine is linked. Our experience has shown that such groups likewise decide other biological properties of this class of substances. Without stopping here to deal at length with the toxicity experiments it may be said that in general the toxicity of these compounds is determined by such groups. For example, whereas the *o*-nitro benzyl derivative could be given to mice intravenously in amounts up to 500 mg. per kilo, the 2,3-dimethoxybenzyl derivative was found to be fatal in 0.1 of this dose.

SUMMARY.

By the addition of substituted benzyl halides to hexamethylenetetramine, a series of quaternary salts of this base was obtained. These salts represent a new group of organic bactericides. The results obtained in the tests with these substances upon *Bacillus typhosus* have demonstrated the existence of direct relationships between chemical constitution and bactericidal action within the series.

The bactericidal character is directly attributable to the presence of the hexamethylenetetramine nucleus. The degree of the bactericidal action, however, is determined by the position, character, and number of the groups substituted in the benzene nucleus.

By the introduction of the methyl, chlorine, bromine, iodine, cyano, and nitro groups into the benzene nucleus of the parent benzyl hexamethylenetetraminium salt, the bactericidal power of this compound was notably enhanced. The substitution of these groups in the *ortho* position almost invariably resulted in substances which were more active than their *meta* or *para* isomers. The introduction of the methoxy group was without marked effect.

Several substances in which two hexamethylenetetraminium side-chains occurred were found to be the most active of the substances of this series when tested against *Bacillus typhosus*. Comparative tests with other bacterial types demonstrated that these compounds possessed a marked degree of specificity for *Bacillus typhosus*.

THE BACTERICIDAL PROPERTIES OF THE QUATERNARY SALTS OF HEXAMETHYLENETETRAMINE.

III. The Relation between Constitution and Bactericidal Action in the Quaternary Salts Obtained from Halogenacetyl Compounds.

By WALTER A. JACOBS, Ph.D., MICHAEL HEIDELBERGER, Ph.D., AND CARROLL G. BULL, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, March 1, 1916.)

In the course of the biological investigations of the substituted benzyl quaternary salts of hexamethylenetetramine discussed in the previous paper, it was found that the further extension of this chemical type was no longer advisable, in spite of the fact that within this group substances had been found which possessed considerable bactericidal power against *Bacillus typhosus*. This was due partly to the inaccessibility or insolubility of the further preparations of this type which seemed theoretically indicated. Our attention was in consequence turned to the problem of finding a new scheme of chemical procedure which would furnish the possibility of new leads and of broader development. This was found in the readiness with which hexamethylenetetramine as a tertiary base reacted with halogenacetyl derivatives to form soluble quaternary salts with the following general structure,

$$\left.\begin{matrix} R \cdot OC \cdot CH_2 \\ Cl \end{matrix}\right\rangle (C_6H_{12}N_4)$$

in which R might represent the radical of any aliphatic or aromatic primary or secondary amine R′NH, R′$_2$N, R′R″N, or of any alcohol or hydrocarbon. Because of the general nature of this reaction and the practically limitless number of chemical possibilities offered, it was found possible to furnish the most varied chemical groupings

with the hexamethylenetetramine molecule by the use of the halogenacetyl side-chain.[1]

Without anticipating the detailed discussion of the observations to be found in the experimental part, the following remarks may be taken to sum up the principal results of the work presented in this communication.

As a result of experiments on a large number of drugs of this type, the general statement may be made that hexamethylenetetramine, when combined with halogenacetyl compounds in the form of quaternary salts, gives rise to a new group of organic bactericides. Hexamethylenetetramine may, therefore, be described as a definite bactericidogenic group. The extent of the bactericidal power of these substances is, in a measure, controlled by the general character of the molecule or of the particular groups contained therein. In addition, the employment of several species of microorganisms has served the purpose of bringing out many examples of partial specificity, at least one substance with a high degree of specificity being found for each of the four species of bacteria used.

The fact that this specificity shifted from one group of bacteria to another with the change in the chemical composition of the radical added to the hexamethylenetetramine, demonstrated that, in contradistinction to the bactericidogenic character, the specificity relations were determined, not by the hexamethylenetetramine nucleus, but by the added radicals.

From the facts set forth above it will be seen that the program presented in the introductory paper has been partially fulfilled. Thus it was found possible, by starting with the molecular grouping furnished by hexamethylenetetramine, to add the most varied atomic groupings with the aid of the $-CH_2CO-$ group, which served merely as a connecting link. The chemical differences in these added groups caused the wide variations observed in the bacteriological results.

In these observations, which must be regarded only as a beginning, we thus see that once in the possession of a biologically active or potentially active molecular group, it is possible, without produc-

[1] For the chemistry of these substances and the reference to those prepared by others see Jacobs, W. A., and Heidelberger, M., *Jour. Biol. Chem.*, 1915, xx, 685; 1915, xxi, 103, 145, 403, 439, 455, 465.

ing profound chemical changes in the molecule itself, to equip it with a reactive side-chain which in turn will react with other molecular groups and which will furnish the necessary biological properties.

In the present paper we wish to present the results of the bactericidal tests performed with these preparations upon *Bacillus typhosus*, streptococcus, meningococcus, and gonococcus. Here, as in the previous communication, the large number of tests which were made necessitated the early adoption of a rough scheme of drug dilutions. For this reason what was said in the former paper regarding the accuracy of the results must be reiterated here. At best the figures given may be considered as rough approximations. In spite of this, however, in many instances pronounced evidence of the influence of constitution on bactericidal action appeared.

EXPERIMENTAL PART.[2]

Technique.—In testing the germicidal efficiency of the compounds to be described below, 0.5 or 1 per cent solutions of each substance, according to the solubility, were made in distilled water and filtered through a Berkefeld N filter. The other dilutions were prepared from this stock solution by the use of sterile distilled water as a diluent.

The series 1: 200, 1: 400, 1: 800, 1: 1,600, 1: 3,200, 1: 6,400, and 1: 12,800 was employed for the tests. In some cases the sparing solubility of the substance necessitated the omission of the lower dilutions.

5 cc. of each dilution were placed in wide mouthed tubes and the temperature was brought to 20°C. To each of these tubes the bacterial suspensions were added.

In the case of the *Bacillus typhosus* 0.1 cc. of a 24 hour broth culture was added to each of the tubes containing the dilutions of the compound. After 3 hours a standard 4 mm. loop of the mixture was plated in order to determine the number of living organisms. The plates were incubated for 48 hours before counting. Another loopful from the same tube was taken at practically the same time and inoculated into tubes containing 10 cc. of plain broth and the tubes were

[2] We are greatly indebted to Dr. Martha Wollstein and to Dr. Louise Pearce who conducted the tests with the meningococcus and gonococcus, respectively. Their familiarity with the cultural conditions of the two microorganisms was of special value and service.

incubated for 48 hours. It was found that at a certain dilution, using the plating method, there was a very abrupt falling off in the number of colonies in the plates. This point was always marked in the broth tubes by clear-cut differences in appearance of the incubated tubes. The organisms in the lower dilution produced marked turbidity and in the next higher dilution remained absolutely clear. It was apparent that from 50 to 80 organisms were necessary to inoculate a 10 cc. broth tube, so that anything below this number would not show in this culture media. On the other hand, each organism in the plates produced a colony. Having found at the beginning of the work that this point of abrupt decrease in the number of colonies, using the plate method, came always at the same dilution indicated by no growth in the tubes, the plate method was no longer used on account of the large number of drugs tested.

In the streptococcus tests one or two drops of a 24 hour bouillon culture of an ordinary hemolytic strain of streptococcus were added to each dilution tube. After 3 hours at 20°C. a loopful was taken from the bottom of each tube and plated in plain agar or blood agar. A bacterial control was run in distilled water and plated immediately after mixing and also at the end of the incubation period. By such a control it was possible to estimate the percentage of killing when complete killing did not occur. The plates were incubated at 37°C. for 18 hours and the results recorded.

In the case of the meningococcus the tests were made by Dr. Wollstein. Average 24 hour growths of the microorganism on sheep serum agar were washed off with 2 cc. of sterile distilled water. 0.5 cc. of this well mixed suspension was added to each tube containing the compound dilution. This was allowed to stand for 3 hours at 20°C. Then 0.2 cc. of each tube was planted on sheep serum agar slants. These were incubated for 48 hours and the readings taken. Controls accompanied each experiment.

For the gonococcus tests conducted by Dr. Pearce an adult strain of the organism was employed. 24 hour growths on ascitic veal agar were washed off with 3 to 5 cc. of normal salt solution. The exact amount of salt solution depended upon the profuseness of the growth. The object was to obtain a decidedly cloudy but not milky suspension of the bacteria. 0.5 cc. of this suspension was then added to the tubes containing the substance dilutions and allowed to stand at

20°C. for 3 hours. 0.2 cc. was then pipetted from the bottom of the tubes and planted on an ascitic veal agar slant and incubated for 48 hours. The readings were then taken. Controls were run in each experiment.

In all the above experiments the tests were run in duplicate.

TABLE I.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	*B. typhosus* killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Chloroacetamide	1,600	800–1,600	800	800–1,600
Chloroacetmethylamide	1,600	800	3,200	1,600
Chloroacetethylamide	800	800–1,600	3,200	6,400
Chloroacetdimethylamide	1,600	1,600	800	1,600
Chloroacetdiethylamide	1,600	1,600	1,600	1,600
Chloroacetpiperidide	200	800		
β-iodopropionamide	+*	+	800	800

*+ indicates growth after exposure to a dilution of 1:200.

The results obtained with the hexamethylenetetramine quaternary salts prepared from chloroacetamide and the chloroacetyl derivatives of the simpler aliphatic amines will be found in Table I. With but few exceptions these substances were found to kill the organisms used for the test in dilutions of at least 1: 1,600 in 3 hours at 20°C. On the whole, but little variation in action, at least of a magnitude which could be detected by the dilution scheme employed, was obtained by the addition of alkyl radicals to the amide nitrogen in the chloroacetamide salt. The exceptions presented by the behavior of the methyl- and ethylamide derivatives toward the meningococcus are worthy of note. With these substances the action was observed to be about four times as great as that of the unsubstituted chloroacetamide salt or its dimethyl derivative. The unusual activity of the compound obtained from chloroacetylethylamine against gonococcus is also noteworthy.

When it is considered that substances of purely aliphatic nature are represented in this series, the bactericidal power observed is quite unusual. Formaldehyde, which is considered one of the most powerful of the aliphatic bactericides, when tested by the same tech-

nique was found to kill *Bacillus typhosus* in a maximum dilution of 1:1,200. In addition, the molecular weights of these substances are approximately ten times that of formaldehyde, so that if the comparison were strictly drawn the observed figures should be multiplied by ten. On this basis they far exceed formaldehyde in molecular bactericidal power. The activity of the substances of this group as bactericides is attributable entirely to the presence in them of the hexamethylenetetramine molecule.[3]

The comparative results obtained against *Bacillus typhosus* by the same technique with other aromatic substances which have been regarded in the past as strong organic antiseptics are given in Table II. Unfortunately the tests were restricted to *Bacillus typhosus*. Among the substances given in Table I and in those to follow, many will be found which are as active or more active than any given in this table.

TABLE II.

3 Hours at 20° C.

Substance.	*B. typhosus* killed in dilution of 1:
Formaldehyde	1,200
Phenol	+*
Lysol	400
Trichlorophenol	800
Tribromo-*p*-cresol	1,600
Tetrabromo-*o*-cresol	1,600
Tribromo-*m*-xylenol	1,600
Gentian violet	3,200

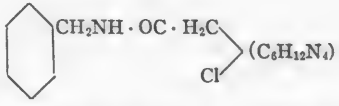
*+ indicates growth after exposure to a dilution of 1:200.

In an attempt to determine what influence might result from the substitution of the halogenacetic acid radical by that of other halogen fatty acids, it was found that the higher α-halogen fatty acid

[3] There is evidence that the hexamethylenetetramine molecule does not always persist as such when its quaternary salts are dissolved in water, but undergoes a decomposition which yields methylene derivatives of the corresponding amines. The relation of these substances to the observed effects we shall make the subject of a subsequent communication.

derivatives failed to react with hexamethylenetetramine. The derivatives of β-iodopropionic acid, however, were found to react smoothly with the base to give quaternary salts. The ineffectiveness of the salt obtained from β-iodopropionamide in particular served to indicate the scant promise offered by the further employment of this acid and that the best results would be obtained by the continued use of the halogenacetyl group.

In the logical development of the above substances of purely aliphatic origin, the effect of the introduction of the aromatic nucleus into the alkyl group situated on the amide nitrogen was studied. The opportunity for this was furnished by the quaternary salts obtained from the chloroacetylbenzylamines possessing the following structural formula:



It was thought that here the usual antiseptic influence of the aromatic nucleus would appear, but, as will be seen from the results presented in Table III, this did not conform to the expectations. In interpreting the results, however, the greater molecular weights of these substances should not be neglected. Nevertheless, in those cases in which the observations fell below 1: 800 the chemical structure alone must be held responsible. Owing to the irregular character of the fluctuations observed it is difficult to deduce from this table any general relationships between the chemical constitution and the bactericidal power. In the case of the streptococcus and gonococcus, however, the introduction of the methyl group seemed to enhance the activity. The methoxy derivatives also appeared to be more effective than the corresponding acetoxy compounds. It is possible that a series of salts prepared from the mono- substituted benzylamines would have afforded a less confusing and more comparable group of substances for study. The difficulty of procuring such material and the pressure of other work were obstacles to the further extension of this chemical type.

On turning to the more easily accessible chloroacetylanilines, a series of substances was obtained which afforded ample opportunity

TABLE III.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	*B. typhosus* killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Chloroacetylbenzylamine.....	1,600*	800	1,600	800
Chloroacetyl-*o*-methylbenzylamine.....	1,600*	3,200	1,600	3,200–6,400
p-acetaminoiodoacetylbenzylamine.....	800*	200	1,600	800
1-methyl-4-acetaminochloroacetylbenzylamine.....	+†	800	800	1,600
1, 2-diacetoxychloroacetylbenzylamine.....	800*	800–1,600	800	400
1, 2-dimethoxychloroacetylbenzylamine.....	1,600*	800	400	800
1-acetamino-4-ethoxychloroacetylbenzylamine.....	800–1,600	800–1,600	400	800
β-acetoxy-α-naphthochloroacetylbenzylamine.....	800	1,600	800	800
β-methoxy-α-naphthochloroacetylbenzylamine.....	1,600	3,200	1,600	1,600
m-carbethoxychloroacetylbenzylamine.....	800	400	400	800
m-carbamidochloroacetylbenzylamine.....	+		3,200	1,600
Diethylaminoethyl ester of *m*-carboxychloroacetylbenzylamine.....	200	800		

* Tests in these cases were made at 37° C.

†+ indicates growth after exposure to a dilution of 1:200.

for ascertaining the influence of the introduction of groups into the benzene nucleus. These substances possessed the following structural formula, in which any group X may occur in the *ortho*, *meta*, or *para* positions:

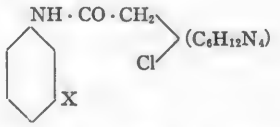


Table IV shows that the linkage of hexamethylenetetramine by means of the chloroacetyl radical with the simpler aromatic amines

TABLE IV.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	*B. typhosus* killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Chloroacetylaniline	800	1,600	1,600	3,200
Chloroacetyl-*o*-toluidine	1,600*	1,600	1,600	1,600
Chloroacetyl-*p*- "	800	800	1,600	3,200
Chloroacetyl-*m*-4-xylidine	800	1,600	1,600	3,200
Chloroacetyl-ψ-cumidine	800	1,600	3,200	3,200
Chloroacetyl-α-naphthylamine	800	800	800	1,600–3,200
Chloroacetyl-β- "	1,600*	800	6,400	6,400
6-chloroacetylaminoquinoline	3,200	3,200	1,600	1,600
Chloroacetyl-*o*-chloroaniline	1,600	1,600	800	3,200
Chloroacetyl-*p*-bromoaniline	1,600	1,600	1,600	3,200
Chloroacetyl-5-iodo-*o*-toluidine	1,600	800	1,600	3,200
Chloroacetyl-*m*-nitraniline	3,200	3,200	3,200	3,200
Chloroacetyl-*m*-nitro-*p*-toluidine	800			
o-chloroacetylaminophenol	1,600	800	800	3,200–6,400
Chloroacetyl-*o*-anisidine	800		3,200	1,600
Chloroacetyl-*p*- "	3,200	1,600		
β-iodopropionyl-*o*- "	+†	200	400	400
Chloroacetylmethylaniline		1,600	800	1,600
Chloroacetyldiphenylamine	400	200	1,600	1,600
p-chloroacetylaminobenzoic ethyl ester	1,600	3,200	1,600	3,200
Chloroacetylnovocaine			1,600	3,200–6,400
o-chloroacetylaminobenzyl alcohol	800	800–1,600	800	800
o- " benzoate	800	3,200	1,600	
o-chloroacetylaminophenyl "	200	1,600	800	3,200
o- " *p*-nitrobenzoate	800	1,600–3,200	1,600	1,600
m-chloroacetylaminoacetophenone		1,600–3,200	800	1,600

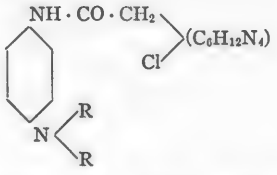
* Tests were made at 37° C.

†+ indicates growth after exposure to a dilution of 1:200.

produced a group of substances possessing definite bactericidal properties. Contrary to the results obtained with the substituted benzyl compounds described in the previous communication, it was found that alterations in the benzene nucleus by the usual substituents did not result in sharp differences in the bactericidal effect, at least of a magnitude which could be revealed by the scheme of dilutions employed. Many instances are to be found, however, in which the ac-

tivity of the simplest member, the salt obtained from chloroacetylaniline, has been definitely improved. Among these may be mentioned the substances obtained by the introduction of the methyl, chlorine, bromine, iodine, and nitro groups. Such chemical variations, however, did not always result in an improvement. In many cases the bacteria were killed in dilutions of 1: 3,200 or even 1: 6,400. On the whole, of the microorganisms employed, the gonococcus was the least resistant towards the members of this group. In the absence of indications of a more decided character or of greater regularity there was little assurance of obtaining more powerful bactericides by the further use of these substituents.

The results, however, assumed a different character by the adoption of a new type of variation within this group of substances. This consisted in the use of the dialkylamino group as a substituent in the nucleus of the parent chloroacetylaniline quaternary salt. These substances were prepared by the reaction of the chloroacetylaminodialkyl anilines with hexamethylenetetramine and possessed the following structure:



in which R may be methyl, ethyl, etc. The bactericidal results are given in Table V.

TABLE V.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	*B. typhosus* killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
p-chloroacetylaminodimethylaniline	+†	800–1,600	1,600	1,600
p-chloroacetylaminodiethylaniline.....		3,200–6,400	1,600	3,200
p-chloroacetylaminodipropylaniline*		6,400	3,200	6,400
p-chloroacetylaminobenzylethylaniline* ..		6,400	6,400	12,800
m-chloroacetylaminodimethylaniline		1,600	400	400

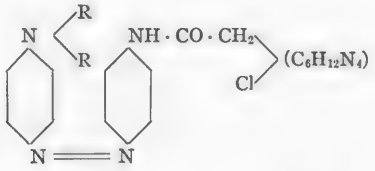
*In these cases, owing to the sparing solubility in water, one mol. of N HCl was used to dissolve the substances.

†+ indicates growth after exposure to a dilution of 1:200.

Our attention was first called to the desirability of developing this series by the apparent partial specificity of the dimethyl compound for the streptococcus as compared with the effect observed upon *Bacillus typhosus*. Later its effectiveness against the meningococcus and gonococcus was found to be equally marked. The later members of the group were obtained by replacing the methyl groups by ethyl, propyl, and benzyl. In these tests *Bacillus typhosus* was unfortunately omitted. We are therefore in no position to state whether this organism is more resistant to these substances as a class.

The regularity of the response to this particular chemical alteration is strikingly shown by these results. A progressive improvement occurred in the bactericidal action upon all three species of bacteria upon proceeding from the dimethyl to the diethyl, dipropyl, and finally the benzylethyl derivatives, and this in spite of the constant increase in molecular weight. If the increase with each dilution had not been so great, it is probable that all the columns of the table would have shown the regularity of the gonococcus results. The striking feature of the observations is the magnitude of the effect produced by such slight alterations in a complicated molecule. The inferior results obtained with the *meta*-dimethylamino compound, the last in the table, would indicate that the relative positions occupied by the substituents in the nucleus are modifying factors.

The efficacy of these groups was still further demonstrated by their use in another class of substances obtained by the addition of hexamethylenetetramine to chloroacetylaminoazobenzene derivatives, in which the base was attached by the chloroacetylamino side-chain to one nucleus and the dialkylamino group to the other as presented in the following formula:



In Table VI the salt obtained from chloroacetylaminoazotoluene, which contains no dialkylamino group, is first given as an

TABLE VI.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	*B. typhosus* killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
p-chloroacetylaminoazotoluene	3,200	3,200	3,200	1,600
p-chloroacetylaminobenzeneazo-*p'*-dimethylaniline*	800	12,800	3,200	3,200
p-chloroacetylaminobenzeneazo-*p'*-diethylaniline	+‡	12,800	1,600	800
p-chloroacetylaminobenzeneazo-*p'*-dipropylaniline*		12,800	800	800
p-chloroacetylaminobenzeneazo-*p'*-benzylethylaniline*		12,800	1,600	800
Benzeneazo-*m*-chloroacetylaminophenol†		3,200		

* In these cases 1 mol. of N HCl was employed to dissolve the substance.

† Solution made by the use of 1 mol. N NaOH.

‡+ indicates growth after exposure to a dilution of 1:200.

object for comparison.[4] The action of this substance upon the different species of bacteria was fairly uniform. The introduction, however, of the dimethylamino group into that position in the molecule farthest removed from the location of the hexamethylenetetramine nucleus resulted in a marked difference. But slight alteration, if any, was observed in the meningococcus, a slight improvement towards the gonococcus, and a considerable reduction in the action upon *Bacillus typhosus*. With the streptococcus, however, the change was profound. The action was increased at least fourfold. The efficacy of this type of chemical modification against the streptococcus was still further confirmed by the replacement of the dimethyl group by the diethyl, dipropyl, and benzylethyl groups. These variations produced compounds which, in spite of the increased molecular weight, exhibited a degree of action of the same order. On the other hand, when tested against the other organisms they were found to be bactericidally less active than the dimethyl compound. We have here an interesting instance of specificity for streptococcus.

[4] The simpler chloroacetylaminoazobenzene derivative could not be conveniently used because of its sparing solubility in water.

Here again the hexamethylenetetramine molecule was found to be a factor in bringing out this effect. This was directly demonstrated in the following manner: *p*-aminobenzeneazodiethylaniline may be considered as the third substance mentioned in the table deprived of hexamethylenetetramine and the $-CH_2CO-$ radical which serves only as a connecting link. This dye was found to kill the streptococcus in a maximum dilution of 1:3,200, an effect which, though marked, is still but one-fourth of the result obtained with its hexamethylenetetraminium salt.

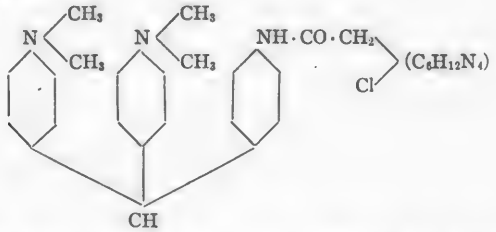
TABLE VII.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	*B. typhosus* killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
p-chloroacetylamino-*p'*,*p''*-tetramethyldiaminotriphenylmethane (*p*-chloroacetylaminoleukomalachite green)*	400	12,800	200	200
o-chloroacetylamino-*p'*,*p''*-tetraethyldiaminotriphenylmethane*		12,800	6,400	1,600
p-chloroacetylamino-*p'*,*p''*-tetraethyldiaminotriphenylmethane*		51,200	1,600	1,600
Chloroacetyltriphenylamine		3,200–6,400	800	800

* In these cases 1 mol. of N HCl was employed to dissolve the substance.

The group of substances given in Table VII headed by the hexamethylenetetramine quaternary salt of chloroacetyl-*p*-aminoleukomalachite green,

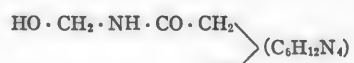


afforded the opportunity of still further testing the value of dialkylamino compounds against the streptococcus. This salt, as well as its homologs, displayed a marked specificity for this microorganism, kill-

ing it in a dilution of at least 1: 12,800, whereas the effect on the other forms was relatively weak. In the next two compounds the methyl groups were changed to ethyl groups, and in one case the chloroacetylamino radical was shifted to the *ortho* position. The dilution of 1: 12,800 was usually the highest dilution employed for the tests in the routine procedure, but fortunately the experiments performed with the third substance were carried further. This preparation was found to kill the streptococcus even in a dilution of 1: 51,200, making it probable that the first and second substances also would have been found to kill above 1: 12,800.

That here also the hexamethylenetetramine molecule is an essential factor was proven as follows. The first substance given in the table when deprived of this base and its connecting group is nothing else than *p*-aminoleukomalachite green. This substance required a concentration of at least 1: 800 to kill the streptococcus in 3 hours. In other words, the hexamethylenetetraminium salt derived from it was at least sixteen times more active.

In the course of the present work our interest was centered for a time in the study of the biological properties of the hexamethylenetetramine quaternary salts obtained from the chloroacetylalcamines. Our attention was attracted first to this group of substances by the powerful bactericidal properties of the simplest representative, that obtained by the addition of hexamethylenetetramine to chloroacetylaminomethanol.[5]



This substance, which possesses the above structural formula, is the first given in Table VIII. It is seen to possess a marked action against all the species used with the exception of the streptococcus. Because of the unusual effectiveness of this product it was hoped that its suitable chemical variation might lead to a series of very active substances.

[5] This substance was first prepared by Einhorn and Göttler (*Ann. d. Chem.*, 1908, ccclxi, 150), who also recognized its antiseptic properties.

TABLE VIII.

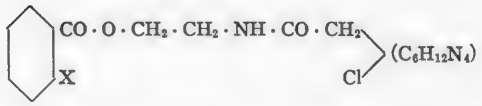
3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	*B. typhosus* killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Chloroacetylaminomethanol	3,200	400–800	6,400	6,400
Iodoacetylaminoethanol		400	1,600	800
Chloroacetylaminoisopropanol		200		
δ-chloroacetylamino-*n*-butanol		400		
β-chloroacetylamino-γ- "		800		
γ-chloroacetylamino-β-methyl-β-butanol		400		
α-phenyl-α-oxy-β-chloroacetylaminoethane		1,600		1,600
β-phenyl-β-oxy-α-chloroacetylaminopropane		800		
Chloroacetylaminoethyl ether		1,600		
Chloroacetyl-*o*-methylphenoxyethylamine		800		

The chemical development of this substance was attempted in two ways: first, by the replacement of its methanol group by the ethanol, propanol, butanol, etc., radicals; and second, by the acylation of the methanol hydroxyl group with various acid radicals. Inthe latter scheme, however, chemical difficulties were encountered which compelled the use of its homologs, in particular the ethanol derivative, as the basis for the study of the effect of acylation.

Table VIII presents the behavior principally towards streptococcus of the substances obtained by the first method of chemical variation. The results show that the first member of the group, the salt obtained from chloroacetylaminomethanol, is the most powerful, so that no bactericidal increase was to be gained by such a chemical procedure.

Quite a different result was obtained by the use of the second scheme of chemical variation, as will be seen in Table IX. Unfortunately the inaccessibility of the acylated methanol derivatives made impossible a direct comparison of the effect of acylation upon the chloroacetylaminomethanol salt itself. The results must therefore be referred to the parent unacylated alcamine compound in question for a strict comparison. The structural formula of this group of substances may be represented as follows, X being any substituting group:



A glance at the table will show that we have here another group of hexamethylenetetraminium salts with strong bactericidal properties surpassing in this respect the parent chloroacetylalcamine compound

TABLE IX.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Chloroacetylaminoethyl benzoate	800	400	1,600–3,200
" *o*-methylbenzoate	1,600	3,200	1,600
" *p*- "	3,200	1,600	800
" β-naphthoate	3,200	6,400	6,400
" *o*-nitrobenzoate	800	3,200	1,600
" *p*- "	6,400	6,400	3,200
" *p*-methoxybenzoate	3,200		
" acetylsalicylate	800		
" *p*-diethylaminobenzeneazo-*p'*-carboxylate	6,400–12,800	400	400
Chloroacetylamino-γ-propyl *p*-nitrobenzoate	1,600		
" -γ- " *p*-methoxybenzoate	1,600–3,200	800	1,600–3,200
Chloroacetylethylaminoethyl *p*-nitrobenzoate	3,200		
Chloroacetylphenylaminoethyl *p*- "	800		3,200–6,400

By the introduction of the simplest aromatic acid, benzoic acid, the bactericidal power of the parent iodoacetylaminoethanol salt was doubled, except for the meningococcus. The use of the substituted benzoic acids, such as the methyl, nitro, and methoxybenzoic, and naphthoic acids, in most cases still further improved the action. In the case of the nitrobenzoates the *para* compound seemed more effective than its *ortho* isomer. With the methylbenzoyl derivatives the *para* compound was also more active towards the streptococcus than its *ortho* isomer. With meningococcus and gonococcus the reverse was the case. The specificity of the *p*-diethylaminobenzeneazo-*p'*-carboxylate for streptococcus was to be expected from the results already discussed in connection with Table VI. This substance possesses the

diethylamino group. With the few acids studied, the best results were obtained with the *p*-nitrobenzoyl and β-naphthoyl compounds. The results yielded by the use of other alcamines, such as aminopropanol, ethylaminoethanol, etc., would seem to indicate that the optimum effect is to be obtained with the aminoethanol series.

In this group of substances but a few representatives were made and tested. By the use of numerous other acids a much broader series might be developed for study with the possibility of obtaining more active preparations. However, the observations obtained with this small group of substances serve to demonstrate again to what extent the bactericidal effect may be altered by relatively small changes in the molecule. Here, as in the case of the benzylhexamethylenetetraminium salts discussed in the previous communication, the degree of action is determined by the character and position of the substituents in the benzene nucleus. The main source of the bactericidal effect, however, is still the hexamethylenetetramine molecule.

TABLE X.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	*B. typhosus* killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Chloroacetylurea	3,200	800–1,600	1,600	1,600
α-chloroacetyl-β-methylurea	800	400–800	1,600	1,600
α-chloroacetyl-β-benzylurea	400	400	800	1,600
Chloroacetylurethane	400		1,600	800

Still another type of hexamethylenetetramine quaternary salt included in the investigations was that represented by the compound obtained by the reaction of chloroacetylurea with hexamethylenetetramine. In Table X it is seen that for a purely aliphatic substance it exhibited a strong bactericidal power. It was hoped that by turning to the substituted ureas this action might be improved. The experience with the methyl and benzyl compounds, however, showed only a diminution of the activity.

Up to this point the substances which have been the subject of discussion were all quaternary salts obtained from halogenacetylamino compounds. Two other types of substances were included in the

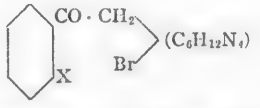
TABLE XI.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	*B. typhosus* killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Chloroacetone	1,600*			
ω-bromoacetophenone	1,600*	3,200	1,600	1,600
p-methyl-ω-bromoacetophenone	1,600	800	800	1,600
p-ethyl-ω- "	800			
1, 2-dimethyl-ω- "	3,200	3,200	3,200	6,400
1, 3-dimethyl-ω- "	1,600		800	1,600
m-nitro-ω- "		1,600		
p-methoxy-ω- "	800	800	3,200	6,400
p-ethoxy-ω- "	800	1,600	1,600	1,600–3,200
p-acetamino-ω- "	800	3,200	1,600	3,200
3-acetamino-4-methyl-ω- "			800	800
3-acetamino-4-tolyl ω-iodoethyl ketone	800	1,600–3,200	12,800	12,800
1, 2-diacetoxy-ω-iodoacetophenone	400	1,600	800	800
β-[ω-bromoaceto]-quinaldine	200	3,200	3,200	3,200

*Tests were made at 37° C.

study in which hexamethylenetetramine was joined by means of the halogenacetyl group first to hydrocarbons and then to alcohols. The first of these groups, which was prepared by the addition of halogen ketones to hexamethylenetetramine, may be represented by the following formula:



The results of the experiments made with these substances are contained in Table XI. The bactericidogenic property of hexamethylenetetramine was again demonstrated. The first member, the salt obtained from chloroacetone, was found to kill *Bacillus typhosus* in a dilution of 1: 1,600, which is again striking for an aliphatic substance. Among the aromatic representatives the majority killed one or another of the species tested in dilutions of 1: 1,600 or more. The behavior of the 1,2-dimethyl-ω-bromoacetophenone and *p*-methoxy-ω-bromoacetophenone derivatives toward the gonococcus and the action of the salt obtained from 3-acetamino-4-tolyl ω-iodoethyl

ketone on the gonococcus and the meningococcus are worthy of note. It is seen that the chemical constitution of the compounds determines in a degree the bactericidal power, but any definite regularity is far from apparent. As in many instances to be seen in the other tables, the result of a particular chemical variation upon the bactericidal power varies according to the organism used for the test. An interesting instance of the influence of the relative positions occupied by substituents in the benzene nucleus upon the bactericidal effect is shown by the differing action of the 1,2- and the 1,3-dimethyl-ω-bromoacetophenone salts towards the meningococcus and the gonococcus. The former substance is four times more active than the latter.

The results yielded by the salts obtained from halogenacetyl esters are given in Table XII. The ease of saponification of this chemical

TABLE XII.

3 Hours at 20° C.

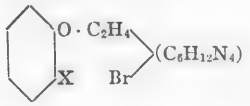
Hexamethylenetetramine quaternary salt of	*B. typhosus* killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Ethyl bromoacetate	400	1,600	800	800
Phenyl "	800	3,200	1,600	3,200
Bornyl "	3,200			
Menthyl "	800	1,600–3,200	1,600	1,600
Ethyl β-iodopropionate	+*	200	200	400
Anisoylglycol chloroacetate	1,600	1,600	1,600	1,600
p-nitrobenzoylaminoisopropyl chloroacetate	1,600	1,600		1,600

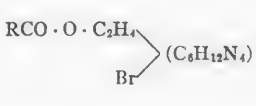
*+ indicates growth after exposure to a dilution of 1:200.

type limited its more extended development. The table demonstrates the bactericidogenic properties of hexamethylenetetramine in this combination also. The relatively low bactericidal power of the β-iodopropionyl derivative is also in line with the results obtained with other derivatives of this acid.

In the course of the work still other connecting groups than the halogenacetyl radical were used in order to combine hexamethylenetetramine in the form of quaternary salts with other molecular group-

ings. Bromoethyl alcohol by virtue of its alcoholic hydroxyl group may combine with acids to form bromoethyl esters or may be considered the mother-substance of the bromoethyl ethers. These bromoethyl derivatives react readily with hexamethylenetetramine, giving the two following classes of salts:





The results obtained with the first of these, the bromoethyl ether salts, are given in Table XIII. It is to be observed that this type was, on the whole, most active against the meningococcus and the

TABLE XIII.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	*B. typhosus* killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Phenyl bromoethyl ether	200	+	400	800
o-methylphenyl bromoethyl ether	+*	400	1,600	1,600–3,200
m- " " "	400		1,600	1,600
p- " " "	400		400	800
α-naphthyl bromoethyl ether	+	800	3,200	12,800
β- " " "	800	1,600–3,200	1,600	3,200–6,400
p-bromophenyl " "	200	+	3,200	1,600
Tribromo-*p*-cresyl " "		3,200	800	800
o-acetaminophenyl " "			400	400
p- " " "			200	200

*+ indicates growth after exposure to a dilution of 1:200.

gonococcus. The partial specificity of the α- and β-naphthol bromoethyl ether salts for the gonococcus is especially noteworthy. The α-compound, which killed the gonococcus in a dilution of 1:12,800, was ineffective against *Bacillus typhosus* in a concentration of 1: 200. These instances, together with the other substances mentioned in the table which were found to kill one or the other microorganism in dilutions of 1: 1,600 or 1: 3,200, still further indicate how general in character is the bactericidogenic property of the hexamethylenetetramine molecule

TABLE XIV.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	*B. typhosus* killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Bromoethyl acetate	+*		800	400
" benzoate	200	400	400	800
" *p*-nitrobenzoate	+		800	800
Bromoethylphthalimide	+	+	400	800

* + indicates growth after exposure to a dilution of 1:200.

In the case of the bromoethyl ester salts (Table XIV) the introduction of the hexamethylenetetramine molecule is seen to be considerably less effective. However, here again the best results were obtained with the meningococcus and the gonococcus. The last substance in the table is not an ester but a bromoethylamino compound. This also was most active against the meningococcus and gonococcus. It would seem from both this series and the previous group of substances that there is something in the chemical nature of the salts obtained from bromoethyl compounds which renders them most active against these two species of bacteria. Although not as marked, this may be considered analogous to the specific effect of the dialkylamino group upon the streptococcus.

It is highly probable that the further development of any of the leads which have been indicated in these papers might eventually furnish more active preparations which would be of chemotherapeutic value.

In conclusion we wish to present the results obtained in a few experiments on the effect of serum and protein on the bactericidal action of several of the compounds mentioned in the preceding tables. It so happened that in these serum-compatibility tests the technique employed was that described in the preceding paper. For this reason the parallel observations made with solutions of the compounds in physiological salt solution are a dilution higher than those to be found in the preceding tables. Table XV presents the results of these tests.

TABLE XV.

Hexamethylenetetramine quaternary salt of	*B. typhosus* killed in 4 hrs. at 37° in a dilution of 1:	
	In physiological salt solution.	In horse serum.
Chloroacetyl-*o*-toluidine	3,200	3,200
Chloroacetylaminomethanol	6,400	3,200
ω-bromoacetophenone	1,600	800
1, 2-diacetoxychloroacetylbenzylamine	1,600	800

It is seen that in the case of the salt obtained from chloroacetyl-*o*-toluidine the action was not inhibited by serum. In the other cases the observed effect was reduced by half in the presence of serum. It is possible that in these cases the apparent inhibition was accentuated by the dilution scheme employed, and that in reality but little relative inhibition occurred. With a few other compounds of this class tested by a different technique a much greater relative inhibition of the bactericidal action was observed. From these experiments we may at any rate conclude that the bactericidogenic hexamethylenetetramine portion of the molecule does not in itself cause serum-incompatibility. The source of this must be sought in the remainder of the molecule.

TABLE XVI.

Hexamethylenetetramine quaternary salt of	Gonococcus killed in 2 hrs. at 20° in a dilution of 1:	
	In aqueous solution.	In 5 per cent sodium caseinate solution.
Chloroacetyl-β-naphthylamine	3,200	1,600
p-methoxy-ω-bromoacetophenone	6,400	6,400
Chloroacetylnovocaine	6,400	6,400
α-naphthyl bromoethyl ether	12,800	6,400
Choloroacetylaminoethyl-*p*-nitrobenzoate	3,200	3,200
3-acetamino-4-tolyl ω-iodoethyl ketone	12,800	1,600

In Table XVI will be found the results of a series of tests in which the substances were dissolved in a 5 per cent sodium caseinate solution. The gonococcus was here used and the technique employed was the same as that described in the other gonococcus tests. In only one

case, the last given in the table, was any marked inhibition to be observed. With the remaining substances mentioned relatively little or no inhibition was observed.

SUMMARY.

The extension of the study of the quaternary salts of hexamethylenetetramine to those obtained by the addition of this base to the most varied types of substances containing aliphatically bound halogen has demonstrated that the introduction of the hexamethylenetetramine nucleus in this manner results in the production of bactericidal substances or enhances the bactericidal action if already present.

In particular it was found possible by the use of the halogenacetyl group, XCH_2CO, as a connecting link, to furnish primary and secondary aliphatic and aromatic amines, alcohols, and hydrocarbons of the most varied character with the hexamethylenetetramine molecule and to study the relation between chemical constitution and bactericidal action in the series of substances so prepared. Because of the variety of chemical types studied, the results are too involved for a detailed summary here.

Many of the substances were found to be very powerful bactericides, and in a number of instances derivatives of purely aliphatic nature were found to possess an unusual bactericidal power.

Bacillus typhosus, streptococcus, meningococcus, and gonococcus were the microorganisms used for the tests, and striking instances of partial specificity were observed. This specificity was found to favor not one species alone, but instances were found in which each of the types of bacilli was shown to be especially susceptible to one or another of the particular types of compound employed. The source of this partial specificity is to be sought not in the hexamethylenetetramine nucleus itself but in the molecule to which it is attached.

The action of some of the substances was tested in the presence of serum or protein and was found to be not at all or only slightly inhibited. In other cases marked inhibition occurred. The factors controlling the serum- or protein-compatibility of these substances are likewise to be sought in that portion of the molecule other than the hexamethylenetetramine.

THE PROTECTION OF PATHOGENIC MICROORGANISMS BY LIVING TISSUE CELLS.

By PEYTON ROUS, M.D., and F. S. JONES, V.M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATE 90.

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The discovery by Metchnikoff of the purposeful character of phagocytosis has so stimulated investigation of the defensive activities of tissue cells that phagocytosis and bacterial destruction are at present almost synonymous in the general mind. The old view that leukocytes provide ingested organisms with a culture medium and a means of transport is now mentioned only in historical résumés. It is known that bacteria may be ingested alive—Metchnikoff himself utilizes this fact in his demonstration of the importance of cells for immunity[1]—and it is known also that a cell may take up too many microorganisms and dying of a surfeit, as one might say, may fail to kill them. But such occurrences are regarded as mere incidents in the process of destruction. The possibility that in certain instances cells not only fail to kill the organisms they ingest, but actively protect them from circulating antibodies seems not to have been considered. Yet the question thus raised has more than passing interest. There are a number of important diseases, among them leprosy, tuberculosis, gonorrhea, Leishmania, caused by microbic parasites which live more or less habitually within tissue cells. The part played by the host cells in the life of such microorganisms and also in the distribution within the body of the diseases they induce has obvious importance.

Unfortunately it is not possible to make direct *in vitro* tests with the microorganisms mentioned and the cells in which they live, for the reason that they fail to give rise to circulating antibodies active

[1] Metchnikoff, E., in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, 2nd edition, 1913, ii, 679.

enough to be suitable for the tests. But the problem can be approached by means of artificial systems. It is possible, for example, to submit leukocytes that have ingested bacteria to a bactericidal serum and observe its effect on the intracellular organisms.

Protection against a Foreign Antiserum.

In a first experiment we have used *Bacillus typhosus*, the leukocytes of the guinea pig, which, according to Pettersson,[2] contain no substances destructive to typhoid bacilli, and the serum of normal rabbits, which is strongly bactericidal for the organism.

Experiment 1.—Washed leukocytes from two sterile, 18 hour, aleuronat exudates of the guinea pig's peritoneal cavity were made into a single thick suspension with Locke's solution, and incubated with typhoid bacilli in the presence of a much diluted mixture of fresh guinea pig serum and antityphoid rabbit serum. The smallest amount of the mixture that would ensure good phagocytosis had been previously determined. Twice this amount was employed. The suspension of typhoid bacilli consisted of four 24 hour slant agar cultures of different strains[3] made up in 80 cc. of Locke's solution.

After 1 hour's incubation films from the phagocytic mixture and from a control mixture without serum were examined, with Manson's stain. Only in the former was phagocytosis observed. It was profuse, though there were still many free bacteria. Now small portions of the mixtures and of other control mixtures were added to large amounts of fresh, normal rabbit serum, and the incubation was continued 2 hours longer. Plating was then done in equal portions of agar. Duplicate tests were made throughout. For the dilutions Locke's solution was used.

As the experiment shows, leukocytes can protect ingested bacilli from the action of a bactericidal antiserum.

The mixture of leukocytes and bacteria subjected to preliminary incubation without serum (Mixture 2), and consequently free of phagocytosis at the end of the first hour, gave many more colonies in the plates made 2 hours after the addition of rabbit serum than did Mixtures 3 and 4 from which leukocytes were absent. This was probably due to the protection of ingested organisms, despite the fact

[2] Pettersson, A., *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1905, xxxix, 423.

[3] These were laboratory strains known respectively as Board of Health, Metchnikoff, Wassermann, and New York Hospital, which had been under cultivation for more than 2 years.

TABLE I.

Mixture.	Locke's solution.	Leukocytes.	Bacteria.	50 % guinea pig serum.	2 % antityphoid rabbit serum.	A.	Colonies per square cm. after 17 hrs.	B.	Colonies per square cm. after 17 hrs.
	cc.	cc.	cc.	cc.	cc.	1 hr.'s incubation 0.3 cc. from each tube was mixed with 2.4 cc. normal rabbit serum; and after 2 hrs.' more incubation 0.5 cc. of this mixture was plated in 6 cc. agar.		Same as A except that 0.3 cc. was mixed with 2.4 cc. Locke's solution instead of serum.	
1	—	0.2	0.2	0.1	0.1		211 294		About 500.
2	0.2	0.2	0.2	—	—		72 49		Innumerable.
3	0.2	—	0.2	0.1	0.1		10 13		Exceedingly numerous.
4	0.4	—	0.2	—	—		25 27		Innumerable.

that the bacteria were all outside the cells at the time the rabbit serum was added. For the rabbit serum itself was able to cause phagocytosis and did actually cause this in the mixture, as the films show. Presumably it brought about the ingestion of some living bacteria which were then protected from its further action by the cells containing them.

The character of the protection was not determined in this experiment. The results of Table I can be interpreted otherwise than wholly in terms of bactericidal action. The serum was agglutinative; and agglutination can of itself produce a reduction in the number of colonies from a bacterial suspension. The leukocytes might have protected the bacilli merely mechanically against clumping and thus have brought about the results seen in the plates. Obviously, for further work a non-agglutinating bactericidal agent was desirable.

An Indicator of Cell Death.

The question came up, furthermore, whether the leukocytes exerting a protective influence were alive. Rabbit serum contains an hemolysin for guinea pig erythrocytes, and might well kill the white cells of this species. To solve the point resort was had to tests with

trypan-blue. Evans and Winternitz[4] state that the dye rapidly colors the nuclei of dead cells but does not stain living ones. The following experiment confirms their observation.

Experiment 2.—A 4 day aleuronat exudate from the pleural cavity of a dog was washed and suspended in Locke's solution. It contained many large mononuclear cells capable of phagocyting rat erythrocytes. A part of the suspension was mixed with rat erythrocytes and dog serum, and incubated for 1 hour, after which an equal bulk of a freshly prepared and filtered solution of trypan-blue (0.02 gm. in 2.0 cc. of Locke's solution) was added and the cells forthwith examined. The nuclei of the majority of them failed to stain. None of the many cells that had phagocyted erythrocytes showed nuclear staining.

Other portions of the original suspension were kept in the ice box for several days and then treated in the same way. Most of the cells now failed to take up the rat corpuscles, and most stained promptly with trypan-blue. Among the few which did not stain were those which had just phagocyted rat cells.

The results of this experiment have been borne out by many subsequent observations involving injury to cells of other types, among them the cells liberated from tissue cultures by digestion of the plasma clot with trypsin.[5] Trypan-blue is a prompt and reliable indicator of whether cells are alive or dead.

Protection against an Inorganic Disinfectant.

The test with trypan-blue showed that rabbit serum is injurious to guinea pig leukocytes subjected to it under the conditions of Experiment 1. At the end of 2 hours' incubation about half the leukocytes were dead, as shown by the nuclear staining, whereas in control specimens incubated in salt solution, they were nearly all alive, very few stained cells being observed. Because of the unfitness of rabbit serum for our work, as thus manifested, it was necessary to find another bactericidal agent, one that would not harm the leukocytes or agglutinate the bacteria. Potassium cyanide proved to have both these qualities.

Clowes[6] was the first to demonstrate the difference in resistance of tissue cells and bacteria to potassium cyanide. As far as we are

[4] Evans, H. M., and Winternitz, M. C., unpublished work, cited by Evans, H. M., and Schulemann, W., *Science*, 1914, xxxix, 443.

[5] Rous, P., and Jones, F. S., *Jour. Exper. Med.*, 1916, xxiii, 549.

[6] Clowes, G. H. A., *Brit. Med. Jour.*, 1906, ii, 1548.

aware, his important observations have not been followed up. He found that tumor cells treated *in vitro* with cyanide in a concentration that killed bacteria remained capable of causing tumors on implantation. Our tests have shown that $\frac{N}{150}$ potassium cyanide in Locke's solution will destroy typhoid bacilli in heavy suspension while failing to kill guinea pig leukocytes, as shown by the trypan-blue test. Furthermore, the bacilli are not agglutinated. In Experiment 3 advantage has been taken of these facts.

Experiment 3.—This experiment closely followed Experiment 1 except that potassium cyanide was substituted for rabbit serum. A watery solution of potassium cyanide, isotonic with 0.95 per cent sodium chloride, was used, diluted with Locke's solution to $\frac{N}{100}$ concentration.

No phagocytosis was observed in Tube 2 after the preliminary incubation; but it was pronounced in Tube 1, though large numbers of bacilli were still free. The leukocytes were tested with trypan-blue at the time of plating and were found to be, in general, still alive, as proved by the fact that their nuclei failed to stain. In more concentrated potassium cyanide solutions they died early as shown by the stain. Some potassium cyanide was carried over into the agar plates but there it was greatly diluted. Control tests with regard to this point showed that the addition to agar of more than ten times the amount of potassium cyanide present in our plates failed to prevent or even to delay the growth of typhoid organisms.

TABLE II.

Mixture.	Locke's solution.	Leukocytes.	Bacteria.	50 % guinea pig serum.	2 % antityphoid rabbit serum.	A.	Colonies per square cm. after 17 hrs.	B.	Colonies per square cm. after 17 hrs.
	cc.	cc.	cc.	cc.	cc.				
1	—	0.2	0.2	0.1	0.1	1 hr.'s incubation then 1.2 cc. $\frac{N}{100}$ potassium cyanide added; 2 hrs.' more incubation and 0.2 cc. plated with 6.0 cc. agar.	144 142	Same as A except that 1.2 cc. Locke's solution added instead of the potassium cyanide.	About 1,500
2	0.2	0.2	0.2	—	—		2 2		Innumerable.
3	0.2	—	0.2	0.1	0.1		0 0		About 1,500
4	0.4	—	0.2	—	—		0 0		Innumerable.

Here there was a marked protection of bacteria by the tissue cells containing them (Table II). The potassium cyanide entirely sterilized the suspensions in which the bacilli were free, whereas in the mixtures in which phagocytosis had occurred, many bacilli survived its action and grew in the plates. One or two colonies developed from Mixture 2, in which there was, supposedly, no phagocytosis because of the absence of serum. But, as is well known, phagocytosis takes place to a slight extent in the absence of serum. The result is readily explained on this basis.

Despite the results of this experiment, the evidence for a protection exerted by living cells specifically is not conclusive. For had the cyanide killed but one phagocyte in every several hundred,—and this may well have happened,—the gross number would be sufficient to permit of an interpretation of the results in terms of a protection exerted by dead phagocytes, not by living ones.

Protection against an Homologous Antiserum.

A second objection to experiments such as the foregoing is that they have little in common with the conditions in the animal body. We have sought to meet both these difficulties by employing red cells as the test object, placing phagocytes which contain them in an hemolytic antiserum derived from an animal of the sort furnishing the phagocytes. With such material it is easy to follow the fate of the ingested corpuscles and to determine by means of the trypan-blue stain whether the individual phagocytes exerting protection are alive or dead. And if the time element be disregarded, one can with good reason liken the conditions as regards the ingested rat corpuscles to those affecting pathogenic microorganisms existing within cells bathed with a lymph containing antibodies.

We have employed rat red cells allowing large mononuclear cells from an exudate in the dog's pleural cavity to phagocyte them, after which an anti-rat dog serum has been added to the mixture. In order to follow the fate of the ingested red cells it has been necessary to know what changes they would undergo when injured within the phagocytes by the hemolytic serum. For the corpuscles cannot lake as they would when free. Fortunately these changes proved easily

recognizable when several red cells were present side by side in a single phagocyte. They have been described and figured by Levaditi[7] and others, who noted them, as we have done, within phagocytes that had taken red cells out of an hemolytic mixture after the hemolysin had attached itself. Under such circumstances hemolysis goes on within the phagocyte with the result that the included corpuscles melt together, as it were, forming one or more large, orange-yellow hemoglobin-containing globules, which on pressure escape from the cell to dissolve instantly in the surrounding fluid. The contrast between these intracellular globules and red cells that remain intact after ingestion is pronounced (Fig. 1).

Experiment 4.—The phagocytes used were mononuclear cells of a 4 day aleuronat exudate in the pleural cavity of a dog. The exudate was washed twice and made into a thick suspension. It contained a very few red cells. The opsonizing serum was derived from the same dog, and so too the 25 per cent suspension of washed dog cells needed for the controls. The serum hemolytic for rat cells came from another dog which received three intravenous injections of rat erythrocytes on successive days and was bled 8 days thereafter. Preliminary examination of the two dogs' blood had shown that they did not agglutinate or hemolyze each other.

The form of the experiment was simple. Rat cells, dog exudate, and a little normal dog serum were mixed together, and, when phagocytosis had taken place, anti-rat dog serum was added to some portions of the mixture, and to others an equal quantity of Locke's solution. Incubation was resumed and from time to time the phagocyted red cells were observed for evidence of destruction. Two preliminary tests were necessary.

(*A*) A determination of the least amount of dog serum which would incite to phagocytosis. The dog serum used for its opsonins contained, as is usual, an hemolysin for rat cells. But the test showed that it could be used to incite phagocytosis in an amount far below the one producing visible erythrocytic change.

(*B*) A test to find out how much anti-rat dog serum was required to hemolyze free rat cells so rapidly that they could not be taken up by dog phagocytes mixed with them. For the anti-rat serum was not only hemolytic but an active stimulant to phagocytosis as well. And, had it been added to the phagocytic mixtures in a quantity which permitted the taking up of cells while hemolysis of them was going on, a proper comparison between the corpuscles in the phagocytes submitted to serum and those submitted to Locke's solution would have been impossible. The results of this test were so interesting that they will be given in detail.

[7] Levaditi, C., *Ann. de l'Inst. Pasteur*, 1902, xvi, 233.

TABLE III.

Mixture.	25 per cent rat red blood corpuscles.	Dog serum.	Exudate.	Anti-rat serum.	Apparent hemolysis.	Real condition as determined microscopically
	cc.	*cc.*	*cc.*	*cc.*		
1	0.1	0.1	0.25	0.5	Complete within 5 min.	No phagocytosis. Complete hemolysis.
2	0.1	0.25	0.25	0.25	Complete. (?)	Considerable phagocytosis. All free red blood corpuscles hemolyzed.
3	0.1	0.25	0.25	0.25 of 50% solution.	+++	Profuse red sediment, of phagocytes enormously distended with red cells. All free red cells hemolyzed.
4	0.1	0.25	0.25	0.25 of 25% solution.	++	

Incubation was for 1 hour at 37°C.

Only in the first mixture was there complete hemolysis and in this all the red cells had suffered destruction within the first 5 minutes of incubation (Table III). In the other mixtures the degree of color of the supernatant fluid at the end of an hour indicated incomplete hemolysis as did the profuse red sediment. But these findings were not due to the serum's lack of hemolytic power, for, as the microscope showed, all the red cells remaining free had been hemolyzed. Many though, had been ingested by cells of the exudate, and thus were protected from hemolysis. The abundant red sediment consisted of phagocytes distended with red cells. Some of the phagocytes had extended only the thinnest layer of glassy cytoplasm over the red corpuscles which stood out, quite unhemolyzed, as knobs on their surface. Such corpuscles were evidently protected from the serum by their intracellular situation. But most of the ingested red cells had been much injured and had coalesced into orange-yellow globules (Fig. 1).

The test made it evident that in order to avoid phagocytosis in the presence of the hemolytic serum sufficient of this serum must be added to cause hemolysis of all the red cells within 5 minutes.

Now the main experiment was proceeded to. The following mixtures were made in a number of tubes.

Mixture 1.—0.1 cc. 25 per cent suspension of rat red blood corpuscles + 0.5 cc. exudate + 0.5 cc. 25 per cent dog serum.

Mixture 2.—0.1 cc. 25 per cent suspension of rat red blood corpuscles + 0.5 cc. exudate + 0.5 cc. 10 per cent dog serum.

At the end of an hour's incubation the mononuclear cells were found to have ingested numbers of apparently unchanged red cells,—from 6 to 25, as a rule.

To some of the duplicate tubes 0.5 cc. anti-rat dog serum was added, to others the same quantity of Locke's solution, and incubation was resumed. At the end of a further hour the preparations were examined for evidence of destruction of the intracellular red cells. None had occurred. The erythrocytes within the mononuclears submitted to anti-rat serum, like those within the phagocytes treated with Locke's solution, were still intact (Fig. 2). But the anti-rat serum had hemolyzed all extracellular erythrocytes and the phagocytes lay in the midst of masses of shadows.

A still more exacting test was carried out, as follows:

Mixture 3.—0.1 cc. 25 per cent suspension of rat red blood corpuscles + 0.5 cc. exudate + 0.5 cc. concentrated dog serum. After 1 hour's incubation 0.5 cc. anti-rat serum was added to some of the tubes, an equivalent amount of Locke's solution to others, and incubation continued for 2 hours more. In this instance the amount of serum used for opsonization caused injury to the red cells, of which some coalesced into globules immediately after their ingestion, though the majority remained intact. And now when the phagocytes full of these globules and of more or less injured and vulnerable cells were submitted to anti-rat serum and to Locke's solution, respectively, for 2 hours, no microscopic differences in their contents were observed. The anti-rat serum had been powerless to enhance the breaking-down of the red cells.

The dog red cells present in the exudate were far too few to constitute a source of error in the findings. Nevertheless, a number of control tests were made with a 25 per cent suspension of dog cells instead of rat cells. They were not phagocyted. In the experimental tests proper, the free dog cells were easily distinguished by their failure to agglutinate or hemolyze in the anti-rat serum.

The results of this experiment were clear-cut. The phagocytes protected red cells within them from the action of a powerful homologous antiserum (Figs. 2 and 3).

Protection a Function of the Living Cell.

The condition of the leukocytes exerting this protective action remained to be determined. Were they perhaps injured by the antiserum, despite the absence from it of agglutinins and hemolysins? The failure of the serum to penetrate could be explained in this way. Or was the protection a function of the living leukocytes and of living ones only? Tests with the material of Experiment 4 threw light on these points.

Experiment 5.—(*A*) Cells of the ultimate mixtures of Experiment 4 were examined with trypan-blue. The phagocytes which had been incubated with

anti-rat serum and those submitted to Locke's solution alike failed to take the stain. Many of the white cells that had failed to ingest red corpuscles showed nuclear staining.

(*B*) The following mixture was made up with the ingredients of Experiment 4:

0.1 cc. Locke's solution + 0.5 cc. washed exudate + 0.5 cc. concentrated anti-rat serum + 0.5 cc. dog serum.

After 1 hour's incubation the cells were separated out with the centrifuge and made up as follows:

0.5 cc. treated cells + 0.1 cc. 25 per cent suspension of red blood corpuscles + 0.5 cc. dog serum.

At the end of an hour profuse phagocytosis had taken place, proving that the leukocytes could not have been seriously injured by the antiserum.

(*C*) Portions of the ultimate mixtures of Experiment 4, in which phagocytosis had occurred, were kept in the ice box at about 2° C. and examined each day. At the end of the first 24 hours the cellular sediment had largely lost its ruddy color. The microscope showed that this was due to diffusion out of the phagocytes of pigment from the ingested red cells. The majority of the leukocytes had now a ground glass appearance. In Mixtures 1 and 2 there could be seen within the phagocytes the intact stromata of red cells from which the hemoglobin had disappeared. In Mixture 3 the stromata were not so clearly visible. In this instance, one will recall, the majority of the red cells were much damaged previous to ingestion. In all the mixtures there were still some phagocytes containing bright red cells, and in Mixture 3 some with orange-yellow globules. Phagocytes containing one or two intact cells and the shadows of others were not observed; but the protoplasm of many phagocytes was stained light orange, due to the seeping out of the hemoglobin from ingested red elements. With the trypan-blue test it was found that the cells containing bright erythrocytes or globules regularly failed to stain. So too did the cells tinted light orange. Practically all the other leukocytes underwent an immediate nuclear staining.

After 3 days in the ice box the results were identical except that living phagocytes were now rare.

This experiment proved that the protection exerted by the phagocytes in Experiment 4 was not due to injury, but on the contrary was associated with active cell life. When the phagocytes died they became permeable, allowing a rapid diffusion outwards of the hemoglobin from the ingested erythrocytes, as well as diffusion inwards of the trypan-blue stain. It seems highly probable from these facts, as well as from common knowledge of the differences in permeability between dead and living tissues, that when phagocytes die they must lose largely if not entirely their protective power.

DISCUSSION.

There are in the literature a number of detached observations which corroborate our findings. Bordet found that "cholera spirilla injected into the blood stream of cholera immune animals are taken up by the leukocytes even before they can be subjected to lysis by the circulating lytic antibodies."[8] And Metchnikoff, Levaditi,[7] Briscoe,[9] and others have shown that red cells injected into the previously immunized animal may be phagocyted before they can hemolyze. But so far as we are aware no attention has been paid to these indications of protection by cells. Such protection had no practical importance in the instances cited because the phagocytes themselves were capable of destroying the organisms they had ingested. In our experiments as well the phagocytes may have possessed this ability. The demonstration of protection by them is not thereby invalidated. For the ability of cells to protect ingested organisms from the action of outside agencies must be considered as entirely distinct from the disposition they ultimately make of these organisms.

It remains to be determined how far the protection of microorganisms by living tissue cells, especially cells incapable of killing the microorganisms, is important in disease processes. The phenomenon may have much to do with the survival in the animal body of organisms such as the leprosy bacillus which is so often found living within cells of the fixed tissues; and it may serve to explain in part the therapeutic difficulties in such instances. It may throw light, furthermore, on the formation of new disease foci at points of injury in individuals of high general resistance. For if an infective agent can be "walled off" from the action of the body fluids by the protoplasm of a single cell containing it, there is no reason why it should not be carried unharmed wherever this cell goes.

CONCLUSIONS.

1. Living phagocytes are able to protect ingested organisms from the action of destructive substances in the surrounding fluid, and even from a strong homologous antiserum.

[8] See Zinsser, H., Infection and Resistance, New York, 1914.

[9] Briscoe, J. C., *Jour. Path. and Bacteriol.*, 1908, xii, 66.

2. There is evidence that the protection by phagocytes is largely if not entirely conditioned on their being alive.

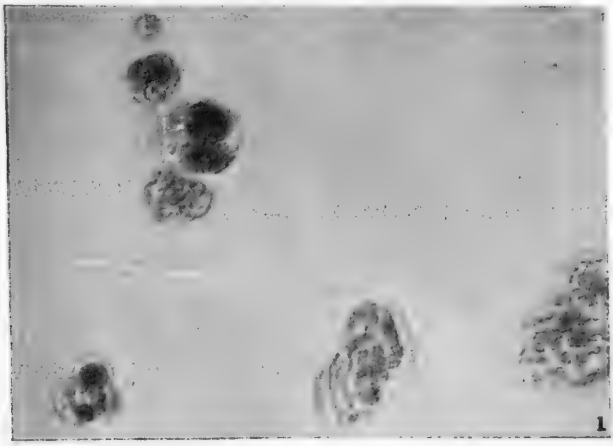
3. These facts should be taken into consideration in the study of diseases caused by infectious agents capable of living within tissue cells.

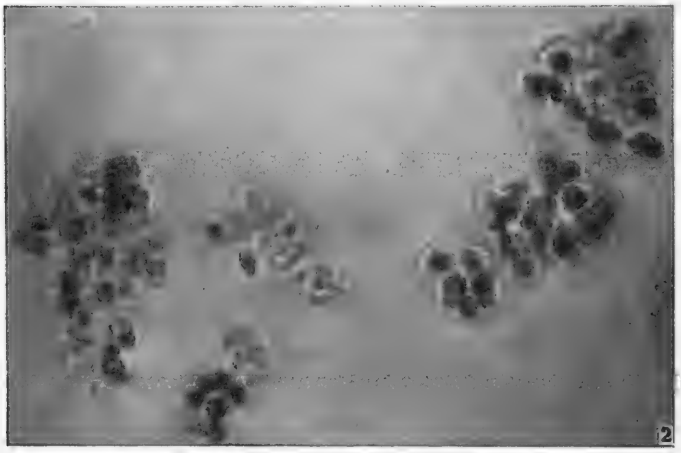
EXPLANATION OF PLATE 90.

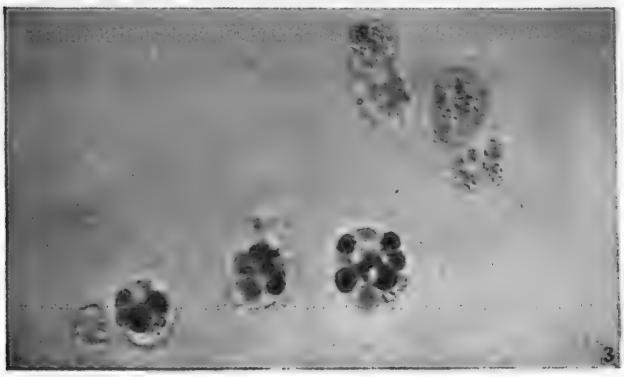
FIG. 1. Red cells hemolyzing within phagocytes. There is a coalescence of the cells into globules. × 625.

FIG. 2. Intact red cells of the rat within dog phagocytes submitted for 1 hour to a powerful anti-rat dog serum. Many of the red cells appear pale because they are out of focus. The only free erythrocytes that have not been laked are a few dog cells. × 625.

FIG. 3. Red cells of the rat still intact within dog phagocytes submitted for 2 hours to a dog serum strong'y hemolytic for rat cells. The shadows of numerous erythrocytes hemolyzed while free are just visible. × 625.







(Rous and Jones: Protection of Pathogenic Microorganisms.)

THE EFFECT OF DIGITALIS ON THE NORMAL HUMAN ELECTROCARDIOGRAM, WITH ESPECIAL REFERENCE TO A-V CONDUCTION.

By PAUL DUDLEY WHITE, M.D., and ROBERT RAY SATTLER, M.D.

(*From the Massachusetts General Hospital, Boston.*)

Plates 91 to 94.

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A study of the effect of digitalis on the normal human electrocardiogram was undertaken by us through a desire to throw more light on the significance of the various grades of heart block not infrequently produced in patients by only moderate amounts of digitalis. Little attention has hitherto been paid to the careful electrocardiographic study of the influence of digitalis on A-V conduction in the normal human heart.

Cohn and Fraser[1] in 1913 reported the study with the string galvanometer of twelve patients with normal cardiac rhythm, four of them without heart lesion. Digitalis in doses equivalent to 2 to 4 gm. of the leaves produced changes in A-V conduction in all the patients. A partial or complete return to the original conduction time was always produced by atropine. In 1914 Cohn[2] reported an investigation with digitalis of patients having an early stage of heart disease with normal mechanism. He concludes that "An effect on conduction may be set down as a usual effect of giving the drug, apart from specific preexisting injury." In our experiment with normal active young adults we have come to the same conclusion and have evidence to show that this defect in conduction is practically entirely due to increased tone and irritability of the vagus.

In this investigation five healthy young male adults were studied by us electrocardiographically. The Cambridge model of the Ein-

[1] Cohn, A. E., and Fraser, F. R., *Jour. Pharm. and Exper. Therap.*, 1913–14, v, 512.

[2] Cohn, A. E., *Jour. Am. Med. Assn.*, 1915, lxiv, 463.

TABLE I.

Table of Measurements of Case 1, Age 29 Years.

	Date.	Electrocardiogram.									Heart rate.	Blood pressure.		Subjective sensations.
		Intervals (sec.).			Amplitudes (10^{-3} volt).									
		P − R	R + S	R to end of T	P_2	R_2	S_2	T_2	T_1	T_3		Systolic.	Diastolic.	
	1915													
Normal (before digitalis).....	Nov. 8	0.164	0.065	0.312	0.1	0.8	0.05	0.15	0.15	0	76	118	78	
After 0.3 gm. digitalis.....	" 10				0.1	0.85	0.05	0.2	0.15	0	71			
" 0.6 " "	" 11				0.1	0.85	0.05	0.1	0.15	−Tr.	68	112	64	
" 0.9 " "	" 12				0.1	0.8	Tr.	0.1	0.15	−Tr.	68			
" 1.2 " "	" 13				0.1	0.85	"	0.1	0.1	0	64	124	72	Headache, anorexia.
" 1.5 " "	" 14	0.164			0.1	0.85	"	0.1	0.15	−0.05	69			
" 1.8 " "	" 15	0.187			0.1	0.8	0.05	0.1	0.15	−Tr.	64	122	72	
" 2.1 " "	" 16	0.186			0.1	0.85	Tr.	−Tr. +0.05	0.05	−Tr. +Tr.	64			Palpitation.
" 2.4 " "	" 17	0.186			0.05	0.8	0	−0.05 +0.05	0.05	−Tr. +Tr.	71	130	70	"
" 2.7 " "	" 18	0.203			0.05	0.85	0	−Tr. +0.05	0.05	−0.05	70			" and anorexia.
" 3.0 " "	" 19	0.205	0.069	0.317	0.1	0.8	+Tr.	−Tr. +Tr.	−0.05 +Tr.	−0.05	77	124	70	Palpitation and nausea.
1 day after stopping digitalis....	" 20	0.222	0.072		0.1	0.9	∓ "	−0.05 +0.05	0.1	∓Tr.	69			Arrhythmia, nausea.
2 days " " "	" 21	0.185			0.1	0.85	0	−0.05 +Tr.	0.1	Tr.	80			Bigeminy at night.

3 days after stopping digitalis....	Nov. 22	0.201			0.1	0.9	Tr.	−0.05 +0.05	−0.05 +0.05	0	86	Acute cold (coryza).
4 " " " "	" 23	0.183			0.1	0.85	0	−Tr. +Tr.	−Tr. +0.05	0	88	Acute cold (coryza).
5 " " " "	" 24	0.217			0.1	0.85	0.2	−0.05 +0.1			77	
7 " " " "	" 26	0.186			0.1	0.85	Tr.	0.05	0.1	−0.05	69	
8 " " " "	" 27	0.172			0.05	0.75	0	0.05	0.1	−Tr.	75	
9 " " " "	" 28											Pauses felt in pulse until noon today.
10 " " " "	" 29	0.170			0.1	0.8	Tr.	0.15	0.1	Tr.	67	
12 " " " "	Dec. 1	0.172			0.1	0.85	0.05	0.1	0.1	0	69	
14 " " " "	" 3	0.164			0.1	0.9	Tr.	0.15	0.2	Tr.	64	
After 3.0 gm. digitalis.												
1 min. after exercise.....	Nov. 19	0.293	0.080		0.1	0.9	"	0.2	0.3	+Tr.	109	
45 " " atropine.....	" 19	0.148	0.077	0.262	0.15	0.85	0	−0.05 +Tr.	−0.05 +0.05	− "	120	
Normal.	*1916*											
1 min. after exercise.....	Jan. 18	0.128			0.3 ±	0.8	0.2	0.3			160	
45 " " atropine.....	" 10	0.138			0.1	0.85	0	0.15	0.15	−0.05	119	
	1913											
Normal control.....	Dec.	0.168			0.1	0.95	0.1	0.2	0.15	0.1	84	
	1915											
" "	Dec. 9	0.165			0.1	0.9	0.1	0.15	0.15	0	63	
	1916											
" "	Jan. 10	0.165			0.1	0.85	0.05	0.15	0.15	Tr.	80	
" "	" 18	0.158			0.1	0.8	∓ Tr.	0.15			75	

TABLE II.

Table of Measurements of Case 2, Age 27 Years.

	Date.	Electrocardiogram.										Heart rate.	Blood pressure.		Subjective sensations.
		Intervals (sec.).			Amplitudes (10^{-3} volt).										
		P−Q	Q+R+S	Q to end of T	P_2	Q_2	R_2	S_2	T_2	T_1	T_3		Systolic.	Diastolic.	
	1915														
Normal (before digitalis).....	Nov. 8	0.161	0.078	0.344	0.15	0.1	1.45	Tr.	0.3	0.15	0.15	67	130	70	
After 0.3 gm. digitalis.....	" 10				0.15	0.05	1.35	"	0.3	0.2	0.1	75			
" 0.6 " "	" 11				0.15	0.05	1.35	"	0.25	0.15	0.1	72	122	70	
" 0.9 " "	" 12				0.1	0.1	1.35	"	0.15	0.05	0.1	79			Palpitation.
" 1.2 " "	" 13				0.15	0.1	1.45	"	0.1	Tr.	−0.05 +0.05	87	116	82	Malaise.
" 1.5 " "	" 14	0.161			0.1	0.1	1.4	"	0.1	0.05	0.05	75			
" 1.8 " "	" 15	0.173			0.1	0.05	1.3	"	0.05	0.1	−0.05	80	102	70	Headache, malaise.
" 2.1 " "	" 16	0.166			0.15	0.1	1.4	"	0.1	0.1	−Tr. +Tr.	73			Malaise.
" 2.4 " "	" 17	0.170			0.1	0.05	1.35	"	−Tr. +0.1	0.1	−Tr. +Tr.	64	112	70	Headache.
" 2.5 " "	" 18	0.172	0.078	0.310?	0.1	0.05	1.35	"	−Tr. +0.1	0.05	−0.05 +Tr.	66	106	70	"
1 day after stopping digitalis..	" 19	0.167			0.15	0.05	1.2	0.05	0.1	0.05	−Tr. +0.05	84			
3 days " " " ..	" 21	0.167			0.1	0.05	1.35	Tr.	0.15	0.1	−Tr. +0.05	75			
5 " " " " ..	" 23	0.172			0.15	0.05	1.4	0	0.2	0.15	0.05	62			
8 " " " " ..	" 26	0.162			0.1	0.05	1.4	Tr.	0.2	0.1	0.05	75			

12 days after stopping digitalis. .	Nov. 30				0.15	0.05	1.35	Tr.	0.2	0.05	0.1	70		
19 " " " " ..	Dec. 7				0.15	Tr.	1.5	0.2	0.3	0.15	0.15	70		
After 2.5 gm. digitalis.														
1 min. after exercise.....	Nov. 18	0.183	0.082	?	0.2	0.1	1.3–1.6	0.15	0.35	0.2	0.15	118		
45 " " atropine.....	" 18	0.144	0.081	0.259	0.15	0.1	1.4	Tr.	−0.1 +0.05	0.1	−0.1	120		
Normal.	*1916*													
1 min. after exercise.....	Jan. 10	0.152			0.2	0.2	1.6+	0.1	0.55			136		
45 " " atropine.....	" 10	0.137			0.15	0.1	1.5	0.1	0.2	0.15	Tr.	128		
Normal control.....	" 10	0.157								0.1	0.1			
" "	" 18	0.155										83		
	1915													
" "	May 10	0.170												

thoven string galvanometer was used with non-polarizable electrodes. Photographic plates containing the three leads of Einthoven were taken before the administration of the drug, at approximately 24 hour intervals during the administration and at intervals of a day or two after the drug was stopped until the electrocardiograms had returned to normal. We have used Caesar and Loretz digitalis leaf in amounts ranging from 2 to 3 gm. at the rate of 0.3 gm. daily. Different amounts of digitalis were used in order to compare the durations of the drug effects. The effects of atropine (0.002 gm. subcutaneously) and exercise (a fast run of about one-quarter of a mile) on the normal and on the digitalized electrocardiograms were studied. Several control records were taken in order to determine the normal range of A-V conduction time in the individuals tested.

Measurements were made by projecting the images on the photographic plates upon a glass screen at a magnification of twenty-five diameters. For this purpose a microphotographic apparatus was used. The electrocardiographic intervals were measured off by calipers on a scale of one-sixtieth of an inch and compared with the measurements of the time intervals. Our maximum error is below 0.01 second. Time intervals of 0.2 second were used instead of smaller intervals, such as 0.04 second, because of the greater accuracy of measurement. In work which one of us did with Lewis[3] on the measurement of P-R intervals in experimental curves it was found that the upstrokes of deflections so often fell upon and were obscured by the time lines separating intervals of 0.04 second that these small intervals were given up and 0.2 second time intervals adopted. Three beats were measured on each plate and their average was recorded in the final tables (Tables I to V).

In addition to the determination of the A-V conduction time as obtained from the P-R or P-Q interval (the latter if a Q is present), the Q-end of S and Q-end of T intervals before and after digitalis have been measured; the digitalis effects on the amplitudes of the electrocardiographic deflections, on the heart rates, on the blood pressures, and on the subjective sensations have been studied. On account of the fact that the tension of the string was not always

[3] Lewis, T., and White, P. D., *Heart*, 1914, v, 335.

accurately adjusted allowance for errors has been made in calculating the curves.

Tables I to V contain the measurements of the A-V conduction times as expressed by the P-Q or P-R intervals, the measurements of the Q-end of S and the Q-end of T intervals, the amplitudes of the deflections, the heart rates, the blood pressures, and the subjective sensations. Table VI contains the effects of exercise on A-V conduction in the normal electrocardiogram. Figs. 1 and 2 show the control electrocardiograms of the five subjects and those taken at the end of the digitalis administration. Fig. 3 shows the atropine and exercise effects on the digitalized electrocardiograms. Figs. 4 and 5 illustrate the phenomenon resulting from digitalis in one of the subjects.

DISCUSSION.

A-V Conduction.

Digitalis caused a delay in A-V conduction in four of our five subjects. In three the lengthening was but slight and hardly greater than the normal range of conduction time in these same individuals (Tables II, III, and IV). In none of these three did the P-R interval equal or exceed 0.2 second. In the first subject (Table I) prolongation of conduction time up to 0.3 second occurred after 3.0 gm. of digitalis, but in no one of the five did the P-R interval increase to as much as 0.2 second after the ingestion of 2.5 gm. or less of digitalis. In every instance after digitalis even when the delay in conduction amounted to more than 0.05 second atropine reduced the P-R interval to less than its original value. The increased vagal action occurring with the rapidly slowing pulse after exercise added to the defect already present after digitalis. Normally we have found that immediately after exercise the A-V conduction time is markedly decreased, more even than it is decreased normally by atropine, 0.002 gm. subcutaneously (Tables I, II, and VI). Our maximum shortening was from 0.158 to 0.112 second. Shortening of the P-R interval after exercise was found by Lewis and Cotton[4] in 1913.

[4] Lewis, T., and Cotton, T. J., *Jour. Physiol.*, 1913, xlvi, p. lx.

TABLE III.

Table of Measurements of Case 3, Age 28 Years.

	Date.	Electrocardiogram.										Heart rate.	Blood pressure.		Subjective sensations.
		Intervals (sec.).			Amplitudes (10^{-3} volt).								Systolic.	Diastolic	
		P–Q	Q+R+S	Q to end of T	P_2	Q_2	R_2	S_2	T_2	T_1	T_3				
	1915														
Normal (before digitalis).....	Nov. 8	0.135	0.104	0.379	0.05	0	1.1–1.25	0.3	0.2	0.25	−0.05 +Tr.	68	122	70*	
After 0.3 gm. digitalis.....	" 10				0.1	Tr.	1.25	0.35	0.2	0.25	−0.05 +Tr.	60			
" 0.6 " "	" 11				0.15	0.05	1.45	0.35	0.1	0.25	−0.1 +0.05	73	125	68	
" 0.9 " "	" 12				0.1	Tr.+	1.3	0.35	−Tr. +0.05	0.15	−0.1 +0.05	73			
" 1.2 " "	" 13	0.134			0.05	0.05	1.3±	0.35±	0.1	0.15	−0.1 +Tr.	70	134	78	
" 1.5 " "	" 14	0.149			0.1	0.05	1.4±	0.35±	−0.05 +0.1	0.15	−0.1 +Tr.	63			
" 1.8 " "	" 15	0.154			0.1	0.05	1.3±	0.3	−0.05+ +0.05	0.15	−0.1 +Tr.	64	140	62	
" 2.1 " "	" 16	0.152			0.1	Tr.	1.35±	0.3	−0.05 +0.1	0.15	−0.1 +Tr.	70			
" 2.3 " "	" 17	0.156	0.108	0.367	0.05	"	1.3±	0.3	−0.05 +0.05	0.15	−0.15	57	126	64	Slight nausea.
1 day after stopping digitalis....	" 18	0.150			0.1	0.05	1.35±	0.35	−0.1 +0.05	0.15	−0.2 +0.1	63			

3 days after stopping digitalis....	Nov. 20	0.149			0.1	Tr.	1.4±	0.35	−0.05 +0.1	0.2	−0.2	75		
5 " " " "	" 22	0.154			0.1	0	1.3±	0.35±	−0.05 +0.1	0.15	−0.15	67		
7 " " " "	" 24	0.148			0.1	0	1.25−	0.35±	−Tr. +0.1	0.2	−0.15 +Tr.	66		
9 " " " "	" 26	0.159			0.1±	Tr.	1.2	0.35±	−Tr. +0.1	0.2	−0.15	74		
12 " " " "	" 29	0.135			0.05	0	1.25	0.35±	0.2−	0.25	−0.05 +Tr.	59		
14 " " " "	Dec. 1				0.05	0	1.2	0.3	0.15	0.2	−0.1	64		
After 2.3 gm. digitalis.														
1 min. after exercise.....	Nov. 17	0.151	0.100	0.290	0.25	0.05	1.0–1.3	0.3–0.45	0.3	0.15	−0.05 +0.1	113		
45 " " atropine.....	" 17	0.144	0.087	0.314?	0.2	0.1	1.3+	0.3	−0.1 +0.05	0.1	−0.2	116		
Normal control.....	Dec. 6	0.144			0.1	0	1.4	0.4−	0.15+	0.25	−0.05+ +Tr.	65		
" "	" 8				0.1	Tr.	1.3±	0.3–0.35	0.2	0.3	−0.05 +Tr.	55		
" "	*1916* Jan. 10	0.141			0.1	0	1.3±	0.35±	0.2	0.25	−0.05 +Tr.	75		

* Blood pressure estimation made Nov. 9, 1915, after 0.1 gm. digitalis.

The U deflection in this subject is unusually pronounced but is unaffected by digitalis. As the T wave flattens it becomes at length level with the U wave.

From the effect of digitalis on A-V conduction in normal hearts it seems to us reasonable to conclude that there is either an abnormal irritability of the vagus or a damage to the conduction tissue itself, if heart block, even a delay in conduction beyond a P-R interval of 0.2 second, occurs in patients after small or moderate amounts of an active preparation of digitalis (for example 1.0 to 2.0 gm. of Caesar and Loretz standardized leaves in 4 to 7 days). If, however, this drug is continued up to and beyond 3.0 gm., a slight defect in A-V conduction then appearing for the first time may be reasonably ascribed to the digitalis and no blame be placed on the conducting tissue.

Arrhythmia.

The greater action, or at least the less balanced action, of the vagus at night slowed the heart rate in two of the subjects (Cases 3 and 1) below the usual rate, in one the pulse falling as low as 48 to the minute; in the other subject the vagal activity was still further evidenced by the occurrence of blocked auricular premature beats—a phenomenon dependent on delay in conduction time (Figs. 4 and 5). This arrhythmia, the only one produced in any of the subjects, began to appear after 3.0 gm. of digitalis had been taken. As far as we are aware it is the first recorded observation of such a result from digitalis. It consisted of an interruption of the normal rhythm by premature ectopic auricular contractions (almost isoelectric in the electrocardiogram) without ventricular response. Polygrams and electrocardiograms of the phenomenon were obtained with considerable difficulty because of the fact that the irregularity almost always occurred late at night, apparently when the influence of the vagus was greatest and tended to disappear if an attempt was made to obtain graphic records. At times it occurred so often as to produce a bigeminy—two normal beats followed by a premature auricular contraction during and after which there was a pause in the pulse. This irregularity first appeared when the influence of digitalis was at its maximum, as shown by the P-R interval (0.222 second) 1 day after stopping digitalis. It occurred off and on for the following 8 days and nights, but since the 9th day after stopping digitalis (3 months ago) it has not once occurred. The subject had never had premature beats so far as known prior to the ingestion of the

TABLE IV.

Table of Measurements of Case 4, Age 24 Years.

	Date.	Electrocardiogram. Intervals (sec.). P–Q	Q+R+S	Q to end of T	Amplitudes (10^{-3} volt). P_2	Q_2	R_2	S_2	T_2	T_1	T_3	Heart rate.	Blood pressure. Systolic.	Diastolic.	Subjective sensations.
	1915														
Normal (before digitalis)	Nov. 9	0.132	0.080	0.303	0.15	Tr.	0.95–1.0	0.35	0.3	0.2	0.1	85	140	100	
After 0.6 gm. digitalis	" 11				0.15	"	1.0	0.3–0.35	0.25	0.15–0.2	0.05±	80	144	100	
" 0.9 " "	" 12				0.15	"	1.0–1.05	0.35±	0.2	0.15	0.05	83			Malaise.
" 1.2 " "	" 13				0.1	"	0.95	0.35	0.2	0.15	0.05	80	122	86	" and headache.
" 1.5 " "	" 14	0.133			0.15	"	1.05–1.1	0.25–0.3	0.2−	0.1+	0.05+	82			Palpitation.
" 1.8 " "	" 15	0.140			0.15	"	0.9–0.95	0.3–0.35	0.2−	0.15	0.05−	83	138	90	Dizziness and anorexia.
" 2.0 " "	" 16	0.150	0.075	0.301	0.15	"	1.0–1.05	0.35±	0.25	0.15	0.1	83	128	90	
1 day after stopping digitalis	" 17	0.147			0.1	0	0.95–1.0	0.3	0.2+	0.2	0.05	81			
3 days " " "	" 19	0.140			0.15	Tr.	0.95	0.3	0.2	0.15	0.05	76			
5 " " " "	" 21	0.142			0.15	"	1.1	0.35	0.25	0.2	0.1	88			
7 " " " "	" 23	0.134			0.15	"	1.05	0.35	0.2	0.1+	0.1−	86			
11 " " " "	" 27				0.15	"	1.05±	0.3	0.25	0.2	0.05	86			
14 " " " "	" 30				0.15	0	1.1±	0.3±	0.3	0.2	0.1±	75			
After 2.0 gm. digitalis.															
1 min. after exercise	" 16	0.155	0.090	0.273?	0.25±	0.05	1.0	0.65–0.8	0.7	0.2	0.4	140			
20 " " atropine	" 16	0.124	0.082	0.263?	0.25	0	0.9–0.95	0.5	0.3	0.15	0.15	138			
Normal control	Dec. 3				0.1	Tr.	0.95–1.0	0.3	0.3	0.25	0.1	79			

TABLE V.

Table of Measurements of Case 5, Age 32 Years.

	Date.	Electrocardiogram.										Heart rate.	Blood pressure.		Subjective sensations.
		Intervals (sec.).			Amplitudes (10^{-3} volts).								Systolic.	Diastolic.	
		P–Q	Q+R+S	Q to end of T	P_2	Q_2	R_2	S_2	T_2	T_1	T_3				
	1915														
Normal (before digitalis).....	Nov. 8	0.138	0.081	0.322	0.2	0.1	1.65±	0.2	0.3+	0.1	0.25+	76	116	82	
After 0.3 gm. digitalis.....	" 9				0.15	0.1	2.0+?	0.2	0.35+?	0.2	0.2+	62			
" 0.6 " "	" 10				0.15	0.1	1.65–	0.15–0.2	0.25	0.15	0.1–	78			
" 0.9 " "	" 11				0.2	0.1	1.65±	0.2–	0.25+	0.2	−Tr. +0.1	72	122	70	
" 1.2 " "	" 12				0.15	0.1	1.7±	0.15	0.2	0.15	−Tr. +0.1	78			
" 1.5 " "	" 13				0.15	0.1	1.6±	0.15–0.2	0.15	0.05	−0.1 +0.1	77	125	70	
" 1.8 " "	" 14				0.15	0.05	1.6–	0.15–0.2	0.15±	0.1	−0.1 +0.05±	67			Slight anorexia, nausea, dizziness.
" 2.0 " "	" 15	0.137	0.080	0.310	0.15	0.1	1.6–	0.15–0.2	−Tr. +0.2	0.1	−0.05 +0.1	78	122	70	Slight anorexia, nausea, dizziness.

1 day after stopping digitalis...	Nov. 16	0.136			0.2	0.1	1.6–1.75	0.25±	−0.05 +0.15	0.05	−0.1 +0.1	88		
3 days " " " ...	" 18				0.15	0.1–0.15	1.7±	0.2±	−Tr. +0.2	0.15	−0.05 +0.1	78		
5 " " " " ...	" 20				0.15	0.1	1.6	0.2	0.25	0.15	−Tr. +0.15	74		
7 " " " " ...	" 22				0.15	0.1	1.7±	0.2	0.2	0.15	−0.05 +0.1	76		
9 " " " " ...	" 24				0.15	0.1	1.45–1.75	0.2	0.15–0.2	0.1	−Tr. +0.05	83		
11 " " " " ...	" 26				0.15	Tr.	1.65	0.2	0.25	0.25	0.05	67		
14 " " " " ...	" 29				0.15	Tr.	1.55–1.8	0.2	0.3+	0.25−	0.1−	76		
After 2.0 gm. digitalis.														
45 min. after atropine.....	" 15	0.123	0.081	?	0.2	0.2±	1.3±	0.2–0.25	−0.1+ +0.1−	0.05	−0.2 +0.05	124		
Normal control.....	Dec. 6 *1916*	0.140										75		
" "	Jan. 9	0.141										75		

drug. For these reasons and because of the fact that it was directly dependent on a certain degree of prolongation of the P-R interval (to 0.295–0.300 second) the arrhythmia can be ascribed to digitalis. The subjective sensations of the irregularity were interesting, for the pause following the ectopic auricular beat could always be foretold by the feeling of the premature auricular systole itself consisting of a wave of fulness rising in the neck and throat. The association of mechanical activity of the auricle with the abnormal deflection in the electrocardiogram is clearly shown in the jugular tracing (Fig. 5 b) taken by Dr. O. F. Rogers, Jr. The production of the premature auricular systole by a mechanical stimulus from the contracting ventricle would at present best explain the fact that the R-P interval is much shorter than the P-R interval just preceding and that it varies little if at all in length.

Amplitudes of Electrocardiographic Deflections.

T Wave.—Cohn and Fraser[1] reported in 1913 their observations that the T wave of the human electrocardiogram was inverted in many of their patients who were under the influence of digitalis. More recently Cohn, Fraser, and Jamieson[5] have shown that digitalis given by mouth to patients began to cause a change in shape and amplitude of the T wave as early as 36 to 48 hours after the administration of the drug had begun, the change increasing as the digitalis was continued and persisting for from 5 to 22 days after the drug had been stopped. It is interesting to note that in all five of our entirely normal subjects, as a result of digitalis the T wave was decreased in amplitude in every lead. There seemed to be no direct connection between the effects of digitalis on the conduction time and on the T wave as indicated particularly well by one subject (Table V), who suffered no defect in A-V conduction but who did show a considerable decrease in the amplitude of T (Fig. 1). We have found that the first electrocardiographic evidence of digitalis and, for that matter, the first evidence, of any sort, of digitalis action is the decrease in the amplitude of the T deflection in the case of normal individuals.

[5] Cohn, A. E., Fraser, F. R., and Jamieson, R. A., *Jour. Exper. Med.*, 1915, xxi, 593.

TABLE VI.

The Effect of Exercise on Normal Electrocardiograms of Cases 1 and 2.

		P-Q interval.	Heart rate.
Case 1.	Normal (before exercise)	0.158	75
	½ min. after exercise	0.112	178
	1 " " "	0.128	160
	2 " " "	0.167	111
	8 " " "	0.176	95
Case 2.	Normal (before exercise)	0.150±	90
	½–½ min. after exercise	0.117	155
	¾ " " "	0.132	145
	1 " " "	0.144	135
	2 " " "	0.150	115
	5 " " "	0.153	99
	10 " " "	0.149	102
	30 " " "	0.156	86

Effect of Exercise and Atropine on the Digitalized T Deflection.

The mechanism by which exercise acts on the heart temporarily removed the traces of the digitalis action on the T wave (Fig. 3), while atropine, 0.002 gm. subcutaneously, actually increased the digitalis effect on this deflection (Fig. 3) although the pulse rate was raised about equally by both procedures. Just the opposite action of these two tests was noted on the digitalized P-R interval.

P_2, Q_2, R_2, and S_2 Deflections.—These showed no clear-cut changes in amplitude as the result of the digitalis in our subjects.

SUMMARY.

Digitalis was given by mouth to five normal young male adults in amounts ranging from 2.0 to 3.0 gm. of standardized leaves in the course of 7 to 10 days. The As-Vs interval was prolonged in four of the five subjects, the greatest prolongation occurring in the case of the subject who received the most digitalis and none at all in one who received only 2.0 gm. There was no prolongation to so great an interval as 0.2 second until 2.7 gm. had been taken. The effects of the digitalis on conduction time began 5 to 6 days after the drug had been started and after 1.5 to 1.8 gm. had been taken. The

effects persisted for 1 to 2 weeks after the drug had been stopped. Atropine removed completely the effect of digitalis on A-V conduction. The slowing heart rate after exercise was accompanied by an enhancement of the defect in conduction. The change in conduction through digitalis was therefore almost entirely, if not entirely, due to increase of vagal tone and irritability.

Digitalis did not affect to an appreciable extent the Q-end of S and the Q-end of T intervals. Exercise and atropine both shortened the ventricular complex Q-end of T while the subject was under digitalis.

The amplitude of the T wave, especially in Lead II, was changed within 48 hours after digitalis had been started, a decrease then beginning which became greater as the drug was continued and which persisted until 10 to 19 days after the digitalis had been stopped. The change in the T deflection preceded by several days the change in conduction time. The T wave, therefore, in the normal subject as well as in the patient gives us the earliest indication of digitalis action.

The amplitudes of P, Q, R, and S were not materially influenced by the amounts of digitalis given.

The pulse rate in two subjects became lower than usual at night as the result of the digitalis; otherwise there was no evidence of vagal action on the sino-auricular node. Blood pressure was uninfluenced by the digitalis. Mild subjective sensations occurred in all the subjects during the administration of the drug.

A curious, hitherto undescribed, digitalis arrhythmia consisting of blocked auricular premature beats occurred in one subject after 3.0 gm. of digitalis had been taken.

Supplementary Note.—A second series of five healthy young male adults has recently (March, 1916) been studied by one of us electrocardiographically during a course of digitalis —2.6 to 3.3 gm. Caesar and Loretz leaf in 9 to 11 days. (The drug was weaker than that used in the previous investigation.) Three subjects showed slight prolongation of the P-R interval but no bradycardia; the other two subjects, who had no delay in A-V conduction, showed a marked total bradycardia after the digitalis (heart rate of 43 in each in the middle of the forenoon). The T_2 deflection of the electrocardiogram

was more or less flattened by the digitalis in all five subjects. Atropine given subcutaneously at the height of the digitalis action depressed the T wave still further in every instance.

EXPLANATION OF PLATES.

In all figures abscissæ equal 0.2 second and ordinates equal 10^{-4} volts.

Where the galvanometer string has been too slack or too tense, the error is seen in the control deflection. The proper correction in the amplitude has been made. Amplitudes have been estimated to 0.05 of a millivolt.

PLATE 91.

FIG. 1. Electrocardiograms (Lead II) of Case 5 (*A*), Case 4 (*B*), Case 3 (*C*), and Case 1 (*D*). The left hand column contains control records; right hand column contains records taken during full effect of digitalis. In the center below is a record of Lead II of Case 1 taken 1 day after digitalis had been stopped and showing the variation in the length of the P-R interval.

PLATE 92.

FIG. 2. The three electrocardiographic leads of Case 2 before and immediately after the course of 2.5 gm. of digitalis. The change in the T deflection in each lead is evident.

PLATE 93.

FIG. 3. Electrocardiograms (Lead II) of Case 4 (*A*), Case 3 (*B*), Case 2 (*C*) and Case 1 (*D*) after atropine (left hand column) and after exercise (right hand column) at the completion of digitalis. In the center below is the record of Lead II of Case 5 after atropine at the completion of 2.0 gm. of digitalis.

PLATE 94.

FIG. 4. Lead II of Case 1 showing blocked auricular premature beats following gradual prolongation of the P-R interval. Electrocardiogram taken 5 days after the completion of 3.0 gm. of digitalis.

FIG. 5. Radial pulse tracing (*a*) and polygram (*b*) of Case 1 taken by Dr. O. F. Rogers, Jr., showing arrhythmia produced by the auricular premature beats which occurred as the result of 3.0 gm. of digitalis. *a'*, evidence in jugular pulse of blocked auricular premature beat. Time interval, 0.2 second.

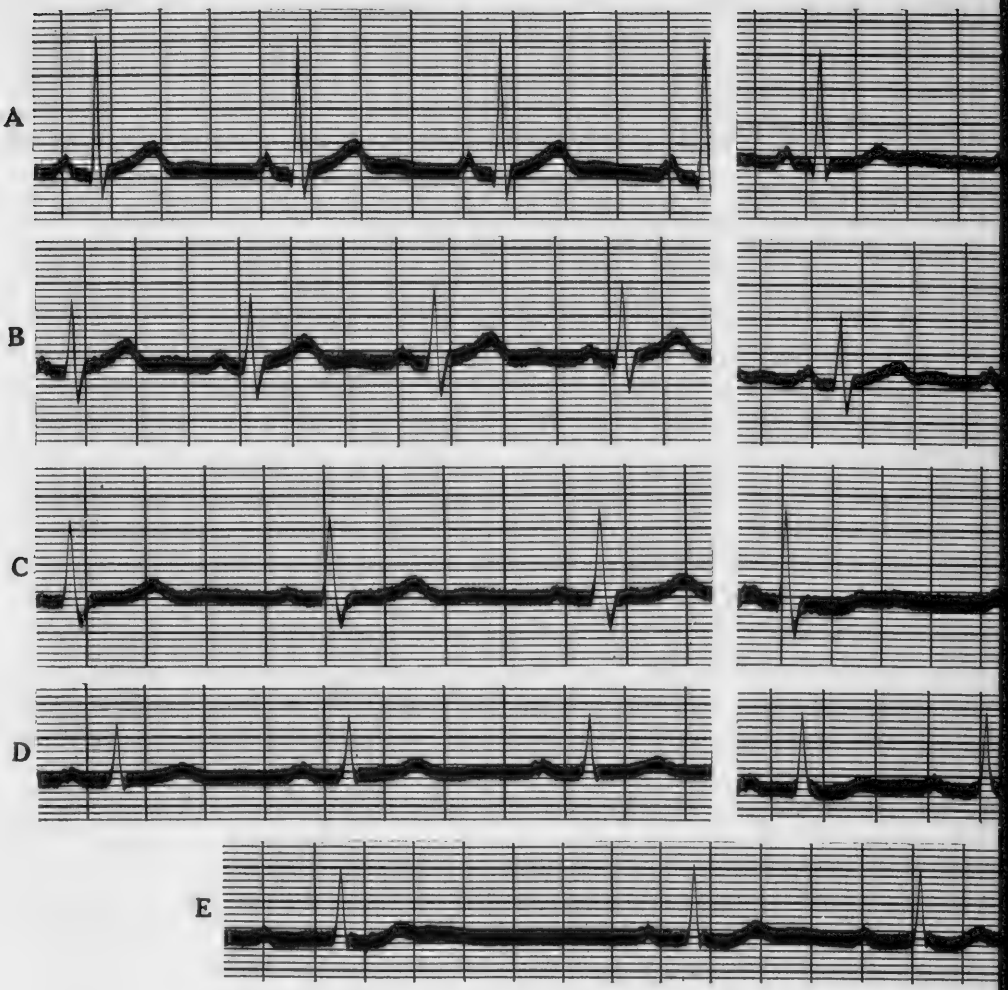

FIG. 1.

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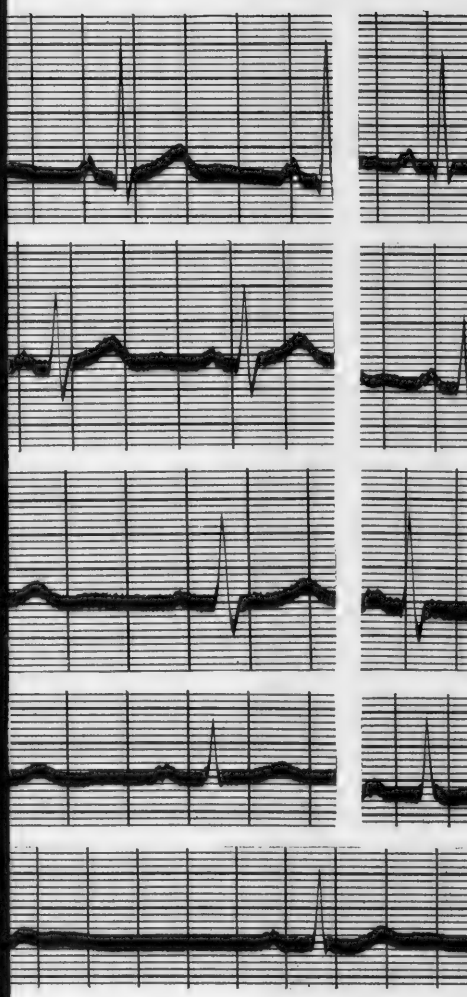
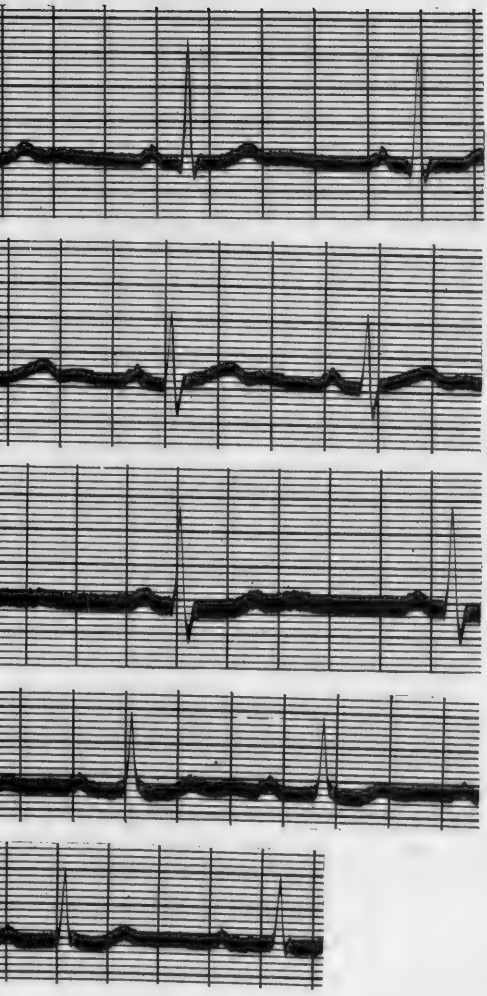


FIG. 1.

PLATE 91.



(White and Sattler: Effect of Digitalis on Human Electrocardiogram.)

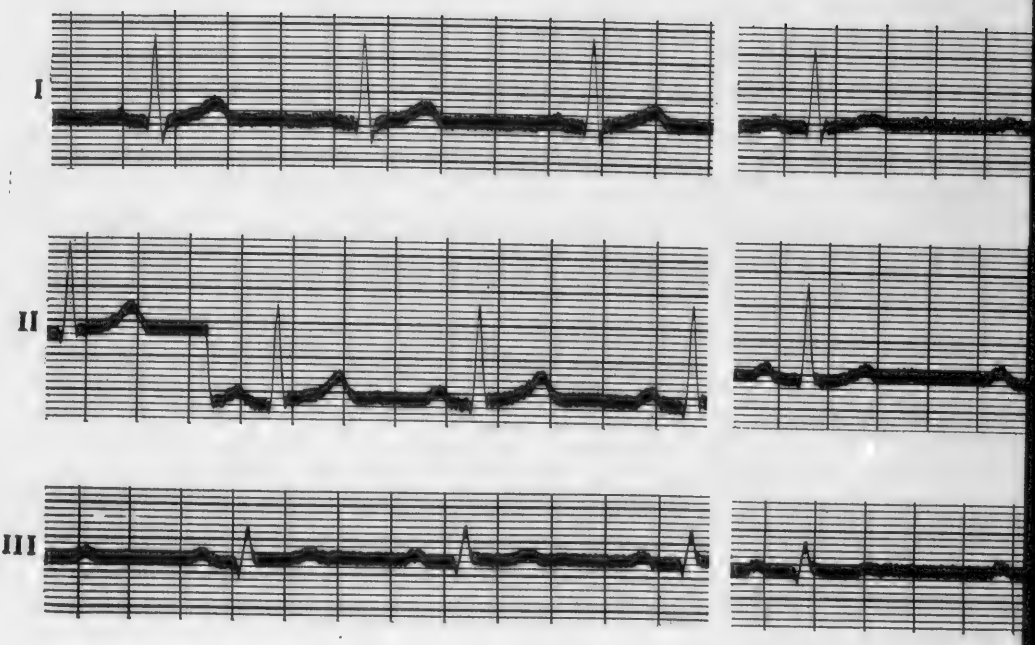


FIG. 2.

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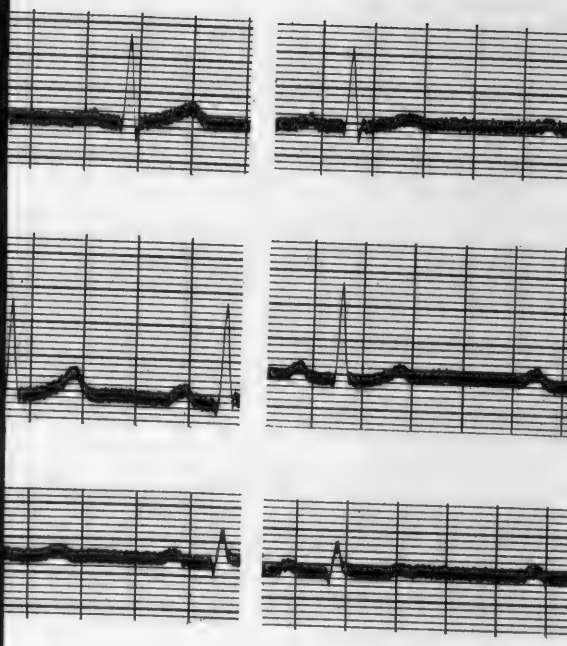
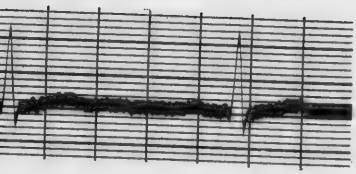
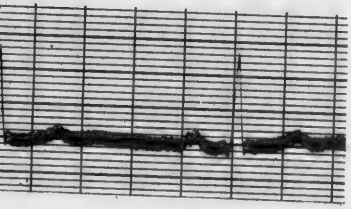
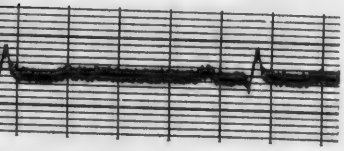


Fig. 2.

PLATE 92







of Digitalis on Human Electrocardiogram.)

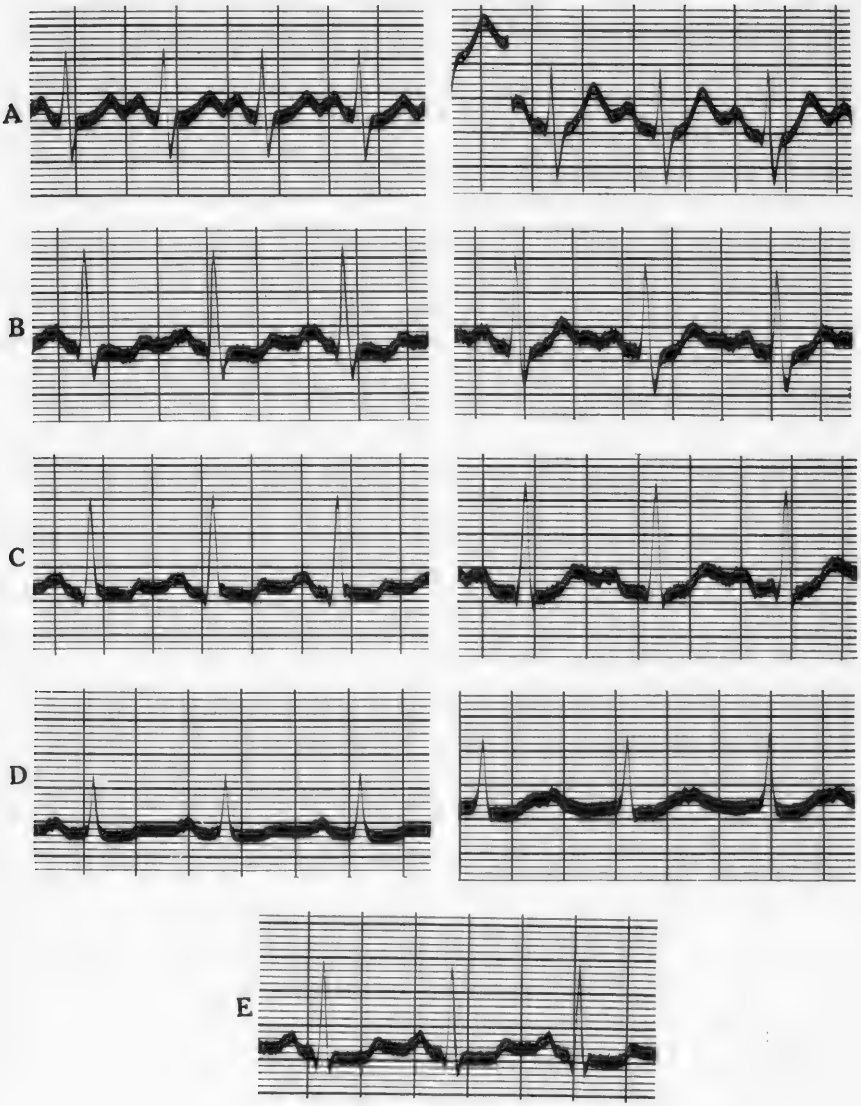


FIG. 3.

(White and Sattler: Effect of Digitalis on Human Electrocardiogram.)

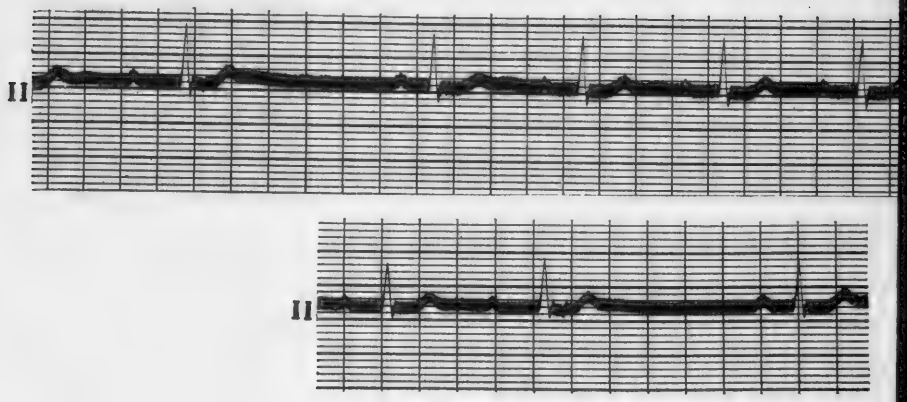


FIG. 4.

FIG. 5a.

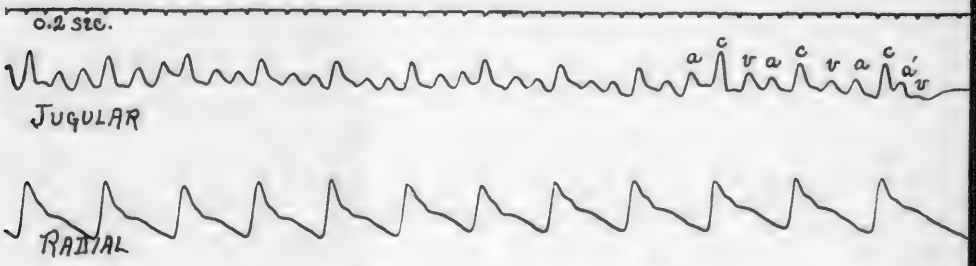


FIG. 5b.

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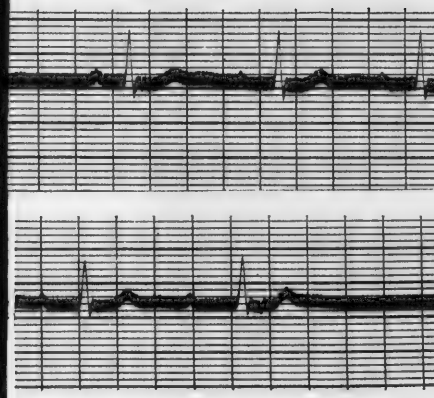


FIG. 4.

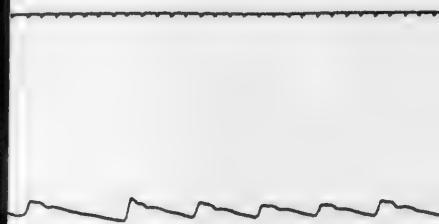


FIG. 5a.

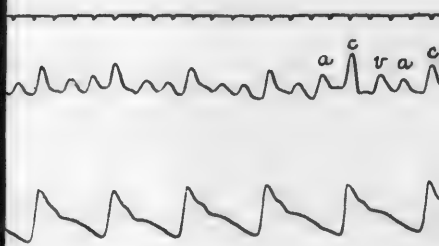
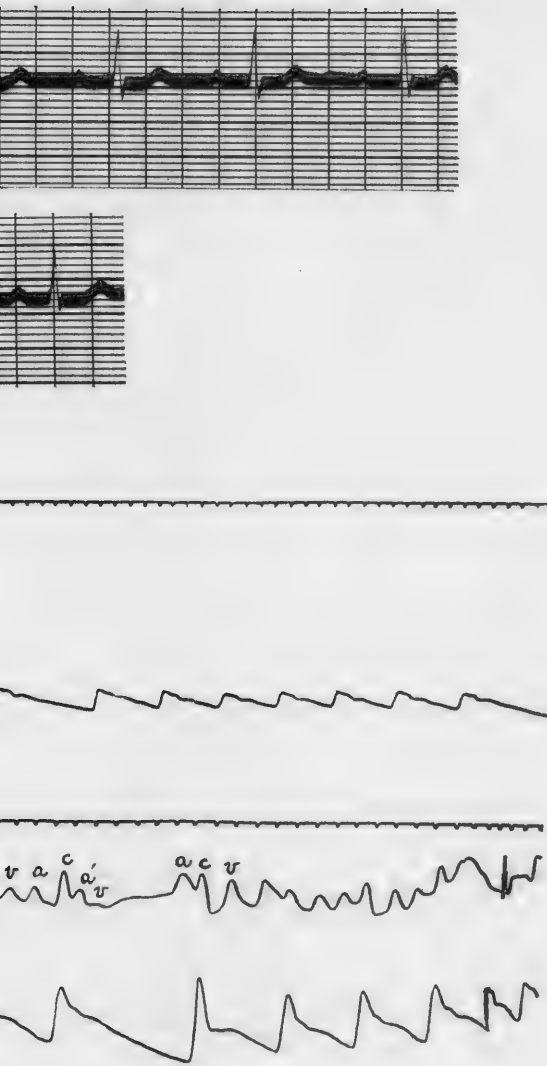


FIG. 5b.

PLATE 94.



(White and Sattler: Effect of Digitalis on Human Electrocardiogram.)

THE INFLUENCE OF TETHELIN, AND OF OTHER ALCOHOL-SOLUBLE EXTRACTIVES FROM THE ANTERIOR LOBE OF THE PITUITARY BODY, UPON THE GROWTH OF CARCINOMATA IN RATS.

By T. BRAILSFORD ROBERTSON, Ph.D., and THEODORE C. BURNETT, M.D.

(*From the Rudolph Spreckels Physiological Laboratory of the University of California, Berkeley.*)

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Preparation of the Substances Employed.

One of us has recently succeeded in isolating the growth-controlling principle (tethelin, from τεθηλώς, growing) from the anterior lobe of the pituitary body. The methods of isolating the substance, its chemical properties and physiological actions, and the evidences of its identity with the growth-controlling principle are contained in articles which have been published elsewhere.[1] Briefly summarized, however, the method of isolating the substance is as follows: The anterior lobes of ox pituitaries are carefully separated from the posterior lobes and connecting parts and stripped of their connective tissue capsules. The tissue is then ground up in a mortar with three times its weight of a mixture of equal parts by weight of anhydrous sodium and calcium sulphates. The mixture is then dried on a water bath until nearly white, returned to the mortar, and thoroughly pulverized.

The pulverized material is then extracted with boiling absolute alcohol in an extraction apparatus (of the Bailey-Walker type) so constructed as to carry out the extraction at or near the temperature of boiling alcohol. The extraction is continued for 48 hours. The solution thus obtained is evaporated under reduced pressure until solid material begins to separate out on cooling. To this con-

[1] Robertson, T. B., *Jour. Biol. Chem.*, 1916, xxiv, 397, 409.

centrated solution is added one and one-half times its volume of dry ether. The substance is thus precipitated and, after washing in large volumes of alcohol-ether mixture containing alcohol and ether in the above mentioned proportions, may be dried over sulphuric acid at low temperatures and pulverized in a dry atmosphere.

The properties of tethelin may be summarized as follows: It is markedly hygroscopic, absorbing water rapidly from moist air. On standing after pulverization in contact with air in the presence of traces of moisture it darkens perceptibly in color and its iodine absorption value decreases. This decomposition is accelerated by warming. If packed in evacuated glass tubes, however, and perfectly dry, it may be heated to 80°C. without any perceptible discoloration.

Tethelin is soluble in water to the extent of about 5 per cent. It is also soluble in alcohol, ether, chloroform, and carbon tetrachloride. It is insoluble in a mixture of one part by volume of absolute alcohol and one and one-half parts by volume of dry ether. It contains 1.4 per cent of phosphorus, and nitrogen in the proportion of four atoms of nitrogen for every atom of phosphorus, two of the atoms of nitrogen being present in amino groups and one in an NH group which is converted into an amino group by hydrolysis with barium hydroxide. It yields unsaturated fatty acid soaps of barium on hydrolysis with barium hydroxide, and among the products yielded by hydrolysis with barium hydroxide followed by hydrolysis with dilute sulphuric acid is found *i*-inosite (hexahydroxybenzene).

Tethelin probably contains an imineazolyl group, and to this extent may be regarded as being related to the physiologically active substances of the posterior lobe of the pituitary body.[2] It is not, however, possessed of the characteristic physiological activity of these substances, relatively large doses administered intravenously to rabbits (50 mg. per kilo of body weight) producing only a very slight transient fall in blood pressure and no diuresis.

The action of tethelin upon the normal growth of mice is identical with the action of the whole anterior lobe.[3] It consists in a marked

[2] Barger, G., and Dale, H. H., *Jour. Physiol.*, 1910–11, xli, 499. Dale, H. H., and Laidlaw, P. P., *Jour. Physiol.*, 1911, xliii, 182. Aldrich, T. B., *Jour. Am. Chem. Soc.*, 1915, xxxvii, 203.

[3] Robertson, T. B., *Jour. Biol. Chem.*, 1916, xxiv, 385, 397.

retardation of the early (preadolescent) growth in weight (subsequent to 4 weeks after birth) and an equally marked acceleration of post-adolescent growth.

In a previous article[4] we have shown that the hypodermic administration of emulsified tissue of the anterior lobe of the pituitary body to rats, either directly into or in localities remote from the tumors, leads to a remarkable acceleration of the growth of the Flexner-Jobling carcinoma, especially during the period of growth between the 20th and 37th days succeeding inoculation. This effect is specific, since similar administrations of liver tissue, during the same period, far from causing any acceleration of the growth of the tumors, actually resulted in a slight but definite retardation of their growth.

In view of these results it appeared of importance to ascertain whether tethelin also reproduces the effect of the whole anterior lobe upon the growth of carcinomata, and to that end the investigations which are about to be described were undertaken. At the same time it seemed advisable to ascertain whether any other alcohol-soluble extractive of the anterior lobe of the pituitary body exerts any action upon the growth of carcinomata. Three such fractions were prepared and their action upon the growth of carcinomata was investigated.

Fraction A.—Tethelin was removed from the alcohol extract by precipitation by ether as described above, and the supernatant fluid was syphoned off from the precipitate and filtered. The ether with part of the alcohol was then distilled off at atmospheric pressure (without exposure to the air) until the boiling point of the mixture reached 78° C. The solution, much diminished in volume, was now cooled by standing in the ice chest for a couple of days. A small quantity of material, slightly soluble in cold alcohol and only partially soluble in ether, was deposited. This was washed in cold alcohol and dried over sulphuric acid. This substance, which behaved like lecithin in forming emulsions with water, composed Fraction A. It probably consisted in great part of a mixture of phospholipins.

Fraction B.—The solution obtained after the separation of Fraction A was evaporated to dryness on a water bath in a vessel provided only with a small exit for the alcohol vapor. The residue was taken up in a small quantity of ether, in which it was completely soluble, and several volumes of acetone were added to this solution. The precipitate was collected on a filter, washed with

[4] Robertson, T. B., and Burnett, T. C., *Jour. Exper. Med.*, 1915, xxi, 280.

acetone and dried over sulphuric acid. This formed Fraction B; it consisted, probably entirely, of lecithins.

Fraction C.—The filtrate from the above was evaporated to dryness in a water bath in a vessel provided only with a small exit for the ether-acetone vapor. The residue, a dark colored substance, gummy when cool and soft and viscous when warmed, was dried over sulphuric acid. This formed Fraction C. It probably consisted largely of ordinary fats and also must have contained all the cholesterol and cholesterol esters originally present in the tissue. On triturating with water it formed an unstable emulsion.

From a number of successive batches of 300 anterior lobes, each weighing approximately 450 gm. (fresh tissue), were obtained the following approximate yields of the above described substances.

	gm.
Tethelin	2.6–3.0
Fraction A	0.3–0.8
Fraction B	4.0–6.0
Fraction C	4.0–6.0

History of the Tumors Employed.

We propagated the Flexner-Jobling carcinoma by inoculation into the axillary region through five generations. The percentage of takes was high, varying between 60 and 90 per cent. Half grown or adult animals were employed to propagate the tumors and also in the experiments enumerated below, with the exception of a small number of younger animals evenly distributed among all the different experimental groups. As stated in our previous communication referred to above, this tumor, non-metastasizing when supplied to us by Dr. Peyton Rous, yielded metastases in the first generation in this laboratory and has continued to do so in succeeding generations.

Influence of Tethelin upon the Growth of Carcinomata.

73 white rats were inoculated in the axillary region with peripheral portions of a large rapidly growing tumor of our 5th generation from Rous's 21st generation. After 21 days 49 of the animals (67 per cent) were found to have well developed tumors. These were divided into two batches. One, consisting of 24 animals was retained as controls, and the 25 individuals comprising the other received, on the 21st, 23rd, 25th, 28th, 30th, and 32nd days after inoculation, 0.6 cc. each of a 5 per cent solution of tethelin in $\frac{M}{6}$ sodium chloride, to which had been added 0.4 per cent of tricresol. The tethelin was injected hypodermi-

cally on the side remote from the tumor. The dose employed (30 mg.) corresponded to three anterior lobes, estimating the tethelin content of each anterior lobe to be 10 mg. No ill effects were observed to follow the administrations.

The tumors were measured through the skin in two diameters at right angles to each other on the dates enumerated below (Table I).

The mean of the two diameters (usually the longest and shortest) was recorded as the average diameter of any given tumor, and the average of these estimates was regarded as the average diameter of the tumors in any given group of animals.

The average diameter of the tumors in the control animals on the 21st day after inoculation was 12.4 mm., that of the tumors in the animals reserved for treatment was 12.1 mm. Calling these initial diameters in each group 100, and referring each of the subsequent measurements to this unit of comparison, we obtained the following results.

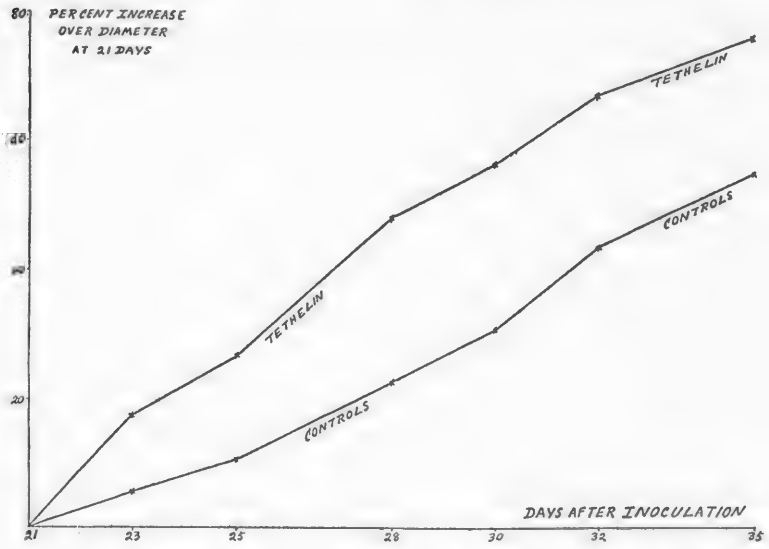
TABLE I.

Days after inoculation.	Diameter of tumors in controls.	Diameter of tumors in animals receiving tethelin.
21	100.0	100.0
23	105.6	117.4
25	110.5	126.4
28	122.6	147.9
30	130.6	156.2
32	143.5	167.0
35	154.9	176.0

These results are depicted graphically in Text-fig. 1. It will be seen that the administration of tethelin caused marked acceleration of the growth of the primary tumors, their linear growth between the first and fifth administration being from two to three times as rapid as that of the controls.

On the 63rd day all the surviving animals (23 of each group) were killed and the viscera examined for metastases. 2, or 9 per cent, of the control animals were found to have developed metastases, while 8, or 35 per cent, of the treated animals had developed metastases. The administration of tethelin, therefore, markedly enhances

the tendency of the tumor to form metastases, the differing result obtained by us with whole anterior lobe administration[4] being probably attributable to the relatively much smaller doses (only 0.5 gm., one-third of an anterior lobe in each dose) employed by us in our previous experiments.



TEXT-FIG. 1. The acceleration of the growth of carcinomata by hypodermic administrations of tethelin.

The Influence of Other Alcohol-Soluble Extractives of the Anterior Lobe of the Pituitary Body upon the Growth of Carcinomata.

140 white rats were inoculated in the axillary region with peripheral portions of a large tumor taken from one of the controls in the preceding experiments; *i.e.*, a tumor of our 6th generation from Rous's 21st generation. After 21 days 105 of the animals (75 per cent) were found to have well developed tumors. These were divided without selection into four batches. One, consisting of 27 animals, was retained as controls; the remaining three groups, consisting of 26 animals each, were treated with Fractions A, B, and C, respectively, on the 21st, 23rd, 25th, 28th, 30th, and 32nd days after inoculation. In each case the substance was administered hypodermically on the side of the animal remote from the tumor. Watery emulsions in $\frac{M}{6}$ sodium chloride solution containing 0.4 per cent of tri-

cresol were employed. The emulsion of Fraction A contained 1.7 per cent of the substance, while the emulsions of Fractions B and C each contained 5 per cent of these substances. The dose administered was in each case 0.6 cc. of the emulsion. No ill effects were observed to follow any of the administrations.

The average diameter of the tumors in the control animals on the 21st day after inoculation was 13.1 mm. The diameters of the tumors in the animals subsequently treated with Fractions A, B, and C were 11.3, 13.4, and 11.1 mm., respectively. Calling these initial diameters in each group 100, and referring each of the subsequent measurements to this unit of comparison, we obtained the following results.

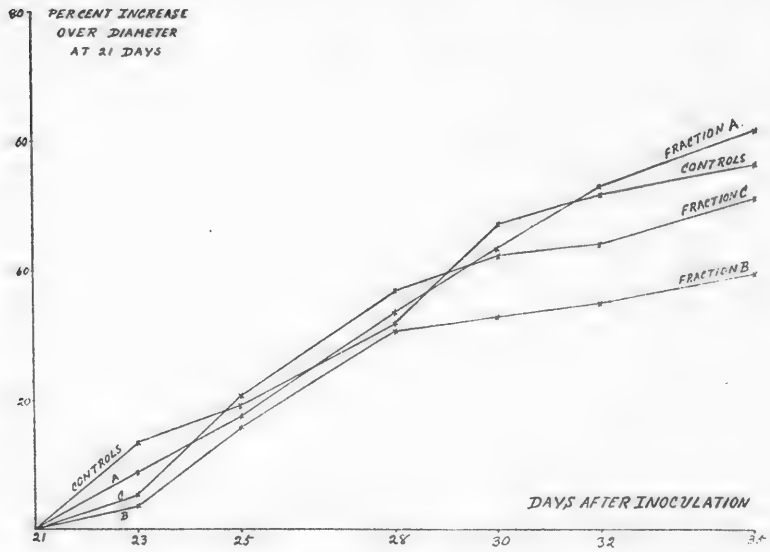
TABLE II.

Days after inoculation.	Diameter of tumors.			
	Controls.	Fraction A.	Fraction B.	Fraction C.
21	100.0	100.0	100.0	100.0
23	113.7	108.8	103.7	105.4
25	119.1	117.7	115.7	120.7
28	132.1	133.6	130.6	136.9
30	147.3	143.4	132.8	142.3
32	151.9	153.1	135.1	144.1
35	156.5	161.9	139.6	151.3

These results are depicted graphically in Text-fig. 2. It will be seen that neither Fraction A nor Fraction C exerted any significant action upon the growth of the tumors. Fraction B, however, caused a marked retardation of the growth of the primary tumors. It will be recollected that Fraction B, consisting as it does of substances soluble in alcohol and in ether, and precipitable by acetone, is composed almost entirely of lecithin. The retardation of tumor growth following its administration is therefore merely confirmatory of our previous finding[5] that administrations of lecithin markedly diminish the rate of growth of the Flexner-Jobling carcinoma in rats. The lecithin employed in our previous experiments, however, was prepared from yolks of eggs by extracting them with ether and adding acetone to this extract. It therefore probably consisted of a mixture

[5] Robertson, T. B., and Burnett, T. C., *Jour. Exper. Med.*, 1913, xvii, 344.

of approximately equal parts of lecithin and cephalin,[6] while the lecithin employed in the experiments just described, owing to the manner of its preparation (extraction of the tissue with alcohol) cannot have contained any admixture of cephalin. Evidently, therefore, lecithin itself is capable of causing the retardation observed in these and our previous experiments. Whether cephalin is capable of



TEXT-FIG. 2. The effect of hypodermic administrations of alcohol-soluble extractives from the anterior lobe of the pituitary body (other than tethelin) upon the growth of carcinomata. Note the retardation due to the lecithin fraction (Fraction B).

exerting a like retardation or whether it only acted in our previous experiments as an inert diluent of the lecithin remains to be ascertained at some future date.

In our previous experiments, moreover, the lecithin was administered by direct injection into the tumors. In the present experiments the lecithin was injected at a locality remote from the tumors.

[6] Stern, M., and Thierfelder, H., *Ztschr. f. physiol. Chem.*, 1907, liii, 370.

Evidently the retarding effect of lecithin is not attributable to a purely local action.

Since Fraction C did not exert an accelerative action upon the growth of the tumors, its content of free cholesterol was presumably small;[5] no analytical determinations have, however, been made.

On the 49th day the surviving animals (twenty-five of each group) were killed and the viscera examined for metastases. The control group and the groups treated with Fractions A and B, respectively, each yielded three animals, or 12 per cent, which had developed metastases. The group treated with Fraction C yielded five animals with metastases, or 20 per cent. It is possible that this slightly enhanced tendency to metastasize may have been attributable to the cholesterol content of this fraction.

CONCLUSIONS.

1. The hypodermic administration of tethelin increases markedly the rate of growth of the primary tumor and the tendency to form metastases in rats inoculated with carcinoma, in this, as in other respects, reproducing the action of the whole anterior lobe of the pituitary body.

2. Other alcohol-soluble extractives of the anterior lobe of the pituitary body, with the exception of the lecithin fraction, exert no appreciable effect upon the growth of carcinomata in rats.

3. The lecithin fraction, as in previously reported experiments in which we employed lecithin obtained from eggs, causes evident retardation of the growth of carcinomata in rats.

In conclusion we desire to express our indebtedness to Mr. Ralston B. Brown, Superintendent of the Oakland Meat and Packing Company, to whose cooperation we owe the supply of pituitary glands which has afforded us the opportunity of carrying out these investigations.

THE INTRAVENOUS INJECTION OF MAGNESIUM SULPHATE FOR ANESTHESIA IN ANIMALS.

By J. AUER, M.D., and S. J. MELTZER, M.D.

(From the Department of Physiology and Pharmacology of The Rockefeller Institute for Medical Research.)

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The effect on animals of intravenous injections of magnesium sulphate was investigated by us from a general experimental point of view about ten years ago.[1] The use of this salt for practical purposes of anesthesia in human beings was first studied by Haubold and Meltzer by the intraspinal method.[2] About two years ago a combination of subminimal doses of magnesium sulphate intramuscularly and ether by intratracheal insufflation was found by the present writers[3] to be effective in animals, and by Peck and Meltzer and also Elsberg and Meltzer in human beings. The use of magnesium sulphate by intravenous injection was in general discouraged by one of us.[4] However, a series of experiments made by the present writers with intravenous injection of magnesium sulphate in cases of experimental tetanus,[5] and the meager but satisfactory experience which Kohn[6] and Straub[7] had with the employment of this method in cases of tetanus in human beings, induced us to take up the experimental study in animals of the employment of magnesium sulphate by intravenous injection for the purpose of producing anesthesia. This was done as a preliminary test for the admissibility of

[1] Meltzer, S. J., and Auer, J., *Am. Jour. Physiol.*, 1905–06, xv, 387.

[2] Haubold, H. A., and Meltzer, S. J., *Jour. Am. Med. Assn.*, 1906, xlvi, 647.

[3] Meltzer, S. J., and Auer, J., *Proc. Soc. Exper. Biol. and Med.*, 1912–13, x, 159; *Zentralbl. f. Physiol.*, 1913–14, xxvii, 632.

[4] Meltzer, S. J., *Berl. klin. Wchnschr.*, 1915, lii, 261.

[5] To be published later.

[6] Kohn, H., *Berl. klin. Wchnschr.*, 1915, lii, 89.

[7] Straub, W., *München. med. Wchnschr.*, 1915, lxii, 341.

studying the exclusive use of intravenous injections of magnesium sulphate as a means of producing, or at least inducing, anesthesia in human beings. We wish to record a few abbreviated protocols of these experiments.

EXPERIMENTAL.

The experiments were made on dogs. The left external jugular vein was exposed under local anesthesia by ethyl chloride, a cannula introduced, and magnesium sulphate in an $\frac{M}{4}$ solution injected through the cannula from a burette. The reaction of the animals was tested in various ways, as indicated in the protocols. When the respiration appeared to be shallow, pharyngeal insufflation[8] was employed either temporarily or throughout the entire experiment. In some instances intratracheal insufflation was given, especially for the purpose of testing the possibility of introducing the intratracheal tube without using any other anesthetic and while the animal was still breathing spontaneously. At the end of the experiment either a small quantity of a calcium chloride or sodium sulphate solution was injected, or no further injection was given.

Experiment 1.—Black and white female fox-terrier; weight 4,600 gm.

10.20. On electric warming pad at medium. Clip hair of neck; used ethyl chloride as local anesthetic for exposing and inserting cannula in external jugular vein.

10.45. Rectal temperature 38.8°C.

10.50. Start infusion of $\frac{M}{4}$ magnesium sulphate into jugular vein.

10.55. Operation completed.

11.00. 5 cc. Lid reflex prompt and strong.

11.04. 14 cc. Slightly restless.

11.05½. Lid reflex prompt; active expiration, of good strength.

11.08. 20.5 cc. Lid reflex prompt and strong. Respiration good, fairly rapid, active expiration.

11.11. 26 cc. Respiration fairly rapid; quiet; lid reflex prompt and strong.

11.13. 33 cc. Respiration slower, with active expiration; no sound. Heart slow.

11.15. Catheter F. 19 inserted into trachea with ease; start air insufflation.

11.17. 40.5 cc. Lid reflex slight.

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[8] Meltzer, S. J., *Jour. Am. Med. Assn.*, 1913, lx, 1407; *Berl. klin. Wchnschr.*, 1915, lii, 425.

11.18. Very slight inspiration, slow rate; able to move head slightly.

11.20. 44.5 cc. Very shallow respiration on stopping insufflation.

11.22. 46.5 cc. Lid reflex fair.

11.23. Occasionally spontaneous, fair expirations during insufflation.

11.24. 50 cc. injected. Stop. Very slight spontaneous shallow respiration; slow rate. Lid reflex slight.

11.25. Injected through venous cannula 15 cc. $\frac{M}{8}$ calcium chloride into jugular vein. Respiration began promptly. Injection lasted about 1 minute. Suture wound after wiping with diluted tincture of iodine. Remove catheter. Animal trots away at once, slightly unsteady at first; wags tail, obeys command.

12.20. Runs about with ease.

2 days later, 10 a.m. Dog lively and active, jumps about, barks, behaves like a normal dog.

Next day. Good condition.

The intravenous injection of an $\frac{M}{4}$ solution of magnesium sulphate was given at a slow rate. After injecting 33 cc. in 23 minutes the intubation for intratracheal insufflation was performed with ease, while usually a great deal of ether has to be given to accomplish this purpose. A total of 50 cc. of magnesium was given in 34 minutes. The spontaneous respiration was then very shallow. After injecting 15 cc. of $\frac{M}{8}$ calcium chloride the respiration recovered promptly and the animal would execute satisfactory voluntary movements.

Experiment 2.—Black and white mongrel, male; weight 5,850 gm.

Preliminary preparations as in Experiment 1.

11.37. Start infusion of $\frac{M}{4}$ magnesium sulphate into jugular vein.

11.41. 9 cc.

11.44. 18 cc. Moves head, lid reflex strong. Barks sleepily.

11.46. 22 cc. Respiration more rapid, shallower; no reaction to pricking skin with needle; lid reflex strong.

11.50. 36 cc. Fair, slow respiration with active expiration. Lid reflex prompt. Occasional slight general motions. Heart slowed.

11.52. 39.5 cc. Respiration slow, moves head.

11.55. 46 cc. Very slow respiration, lid reflex fair; heart slow.

11.57. 50 cc. Respiration very slight; start pharyngeal insufflation. Pulse improved.

12.02. 54.5 cc. Stop insufflation to change stomach tube; no respiration seen.

12.04. 55.5 cc. Heart 24 to $\frac{1}{4}$ minute, small, regular, soft.

12.06. 56.5 cc. Breathes spontaneously; no reaction to pricking skin with needle.

12.09. 61 cc. Tracheal catheter inserted; slow spontaneous respiration, fair depth, some active expiration; lid reflex slight, moves head.

12.12. 64 cc. Legs relaxed; spontaneous respiration fair; lid reflex slight.

12.15. Pulse 27 to ¼, regular, strong (femoral); fair spontaneous respiration. Stop insufflation. 65.5 cc. injected. Respiration gradually improves and becomes good, deep. Respiration moderately slow; start air again.

12.16. Excellent respiration; no lid or corneal reflex. Tongue pink.

12.20. Respiration easy, more rapid; no lid or corneal reflex. 70 cc. injected.

12.22. Moves head; increase magnesium flow slightly.

12.23. 73 cc. Respiration slower, but good depth. Femoral pulse 22 to ¼, respiration good depth.

12.27. No reaction to pricking skin. 78.5 cc. Stop magnesium.

12.28. 8 cc. of $\frac{M}{8}$ calcium chloride into jugular vein. Respiration greatly improved and more rapid. Wound sutured. Placed on floor, holds head up; front legs spread, do not support body; licks jaws; moves tail on pressure. Pays attention to call and wags tail.

12.35. Able to walk about; tail erect.

2.10. Walks about normally.

Next day. Good condition.

The first 50 cc. injected in 20 minutes, about 2.5 cc. per minute, nearly completely abolished respiration. Pharyngeal insufflation was then started and exerted immediately a good effect. From 12.02 to 12.06 only 2 cc. were injected, equal to 0.5 cc. per minute; spontaneous respiration returned, but no reaction to pricking, and intubation of catheter was easily executed. Thereafter the rate of inflow was kept fairly low. Spontaneous respiration was continually present but was generally slow. There was no lid reflex and no reaction to pricking. After injecting about 78 cc. of magnesium in 50 minutes a quantity of 8 cc. of $\frac{M}{8}$ calcium chloride was injected; respiration improved at once. Voluntary movements, however, returned gradually.

Experiment 3.—Male; weight 7,500 gm.

Was fed previous to experiment. Preparation as in previous experiments.

2.27. Rectal temperature 38.7°. Femoral pulse 33 to ¼, regular.

2.33. Start $\frac{M}{4}$ magnesium sulphate into jugular vein.

2.37. 7 cc. Respiration deeper; swallows occasionally.

2.40. Pulse softer, 38 to ¼, regular; respiration more rapid with strong active expiration. 14 cc.

2.41. Vomited yellowish brown fluid.

2.43. Vomited large amount of yellow fluid with masses of meat.

2.44. 22 cc. Lid reflex prompt; occasional moderate struggle.

2.48. 32 cc. Moderate barks. Lid reflex strong.

2.49. 38.5 cc. Respiration chiefly expiratory. Tracheal catheter inserted; animal shows resistance. Catheter F. 21; constant air stream with remission. Lid reflex strong.

2.54. 44 cc. No reaction to pricking skin with needle.

2.55. 45 cc. Stop insufflation; practically only one inspiration and a number of weak abdominal contractions appeared during the intermission. Lid reflex strong. Able to move head moderately. Occasionally a sharp expiratory movement of abdomen. Started intratracheal insufflation again.

3.06. Remove tracheal catheter; start pharyngeal insufflation, tube in stomach.

3.10. 57 cc. Moves head vigorously; femoral pulse regular, soft.

3.13. 59.5 cc. Legs limp. Lid reflex good.

3.22. 73 cc. Open abdomen, rub peritoneum above liver; no motions.

3.23. 76 cc. Lid reflex fair.

3.26. 80 cc. Stop insufflation; no definite respiration, some expiratory contractions. Start insufflation. Abdomen closed.

3.31. 80 cc. No definite respiration on stopping insufflation. Good lid reflex. Pupils wide.

3.35. 90 cc. Stop magnesium. Lid reflex fair. Stop air; 3 slight respirations; start insufflation.

3.38. Slight, slow, shallow respiration on stopping pharyngeal insufflation. Femoral pulse 18 to $\frac{1}{4}$, regular, fair tension.

3.40. 10 cc. of $\frac{M}{8}$ calcium chloride into jugular vein. Deep, slow respirations begin.

3.43. Pulse 29 to $\frac{1}{4}$, regular, strong tension; no reaction to pricking skin with needle. Lid reflex strong; pupils wide. Neck wound closed. Placed on floor, attempts to get up.

3.44. Gets up after a few trials.

3.55. Lies on side; no response to pressure on toes; wags tail; tolerates probe in nose for a short time. When placed on feet walks away fairly steadily, then lies down again.

4.25. As before; no response to pressure on legs; lies on side usually. Raises head on call; walks when placed on feet. Lid reflex prompt; drinks some water. Killed later by chloroform.

This animal was fed about 3 hours before the experiment was started. After 14 cc. of the magnesium solution were injected (in 7 minutes) the animal vomited. This indicates the central action of magnesium sulphate. After 45 cc. were injected (in 22 minutes) there was practically no spontaneous respiration, although the lid reflex was strong and the animal was able to move its head. The failure of the respiration in this case was undoubtedly due to the in-

hibitory action of the magnesium sulphate upon the respiratory center, and not to a paralysis of the motor nerve endings of the respiratory muscles, which, as a rule, remain excitable longer than the other skeletal muscles. After 80 cc. of the magnesium solution (in 58 minutes) the abdomen was opened and the sensitive parts of the parietal peritoneum were rubbed without eliciting any reaction, although the lid reflex was still good. This animal received 90 cc. of the magnesium solution in 62 minutes. 3 minutes after stopping the injection the animal had only slow and shallow respirations. The injection of 10 cc. of $\frac{M}{8}$ calcium chloride deepened the respirations; it exerted also, fairly promptly, a favorable effect upon the general motility of the animal. But the return of reactions to a probe inserted into the nose and to other sensory stimuli was slow.

Experiment 4.—Wolf hound, female; weight 6,700 gm.

Preliminary preparations same as in Experiment 1.

2.42. Start $\frac{M}{4}$ magnesium sulphate into jugular vein.

2.43. 3 cc.

2.46. 10.5 cc. Lid reflex prompt, sustained. Respiration faster; pulse fuller, faster, 33 to $\frac{1}{4}$. Respiratory irregularity of pulse rhythm practically gone.

2.48. 16.5 cc. Respiration 18 to $\frac{1}{4}$, fair depth, slight active expiration.

2.50. 22 cc. Lid reflex prompt, but no longer sustained closure.

2.52. 28 cc. Respiration good, 14 to $\frac{1}{4}$; femoral pulse 30 to $\frac{1}{4}$, good volume and tension; no irregularity.

2.55. 36 cc. Respiration slower; pain sensation of skin abolished.

2.56. 39 cc. Respiration slower, but good depth; pulse irregular. Start pharyngeal insufflation. Stomach tube.

2.58. 46 cc. Lid reflex prompt but slight. Pupils wide.

3.00. 47 cc. Open abdomen and rub diaphragm; no motion of any kind.

3.02. Stop insufflation; very slight shallow respiration; start air; lid reflex a slight flick. Pulse 36 to $\frac{1}{4}$, regular, small volume and tension. Rub peritoneum; no sign of movement; legs limp.

3.07. 51 cc. Lid reflex slightly better; rubbing peritoneum causes no motion of any kind. No knee jerk.

3.08½ 52.5 cc. Stop insufflation; 16 very slight respirations per $\frac{1}{4}$. Rub diaphragm and parietal peritoneum: no motion. Legs limp. Lid reflex fairly sustained now (closure).

3.15. 56.5 cc. Femoral pulse 32 to $\frac{1}{4}$, regular, small, and soft.

3.17. 57 cc. Stop air; no respiration in 35 seconds. Start air. Lid reflex slight; pupils moderately contracted.

3.21. 58.5 cc. Rub peritoneum and diaphragm; slight motion of leg, but no other perceptible movement. Spontaneous respiration noticeable during insuffla-

tion. Stop air; 24 shallow respirations per ¼. Start air. Pulse 32 to ¼, fair volume and better tension now. Lid reflex prompt but weak.

3.25. 59.5 cc.

3.26. Moves leg slightly.

3.29. 61 cc. On rubbing peritoneum and diaphragm no motion, but later made vigorous movements with head and leg. (No apparent relation to stimulus; rubbing repeated; no motion.)

3.31. Some strong movements of head and legs. Lid reflex flick, not sustained. Stop air. Respiration spontaneous, 15 to ¼, good depth. Stop insufflation entirely.

3.33. 63 cc. of magnesium. Stop. Excellent respiration. Suture abdominal wound. Ligate jugular vein and suture wound in neck. No calcium chloride given.

3.36. Placed on floor; attempts to walk; hind legs spread; raises head and looks about.

3.43. Lying on side; placed on legs; walks about; lies down again shortly. Withdraws legs fairly promptly when pressed; walks away when tail is pressed; no sign of pain.

4.00. Walks about. Rectal temperature 36.4°; pulls away leg when toes are pressed.

4.38. Killed by chloroform.

During the first period of the experiment (about 18 minutes) the inflow of the magnesium solution occurred at a rate of about 2.9 cc. per minute. After the injection of 36 cc. (in 13 minutes) skin sensibility was abolished; the respiration, though slower, was good and there was even an occasional struggle. The pharyngeal insufflation was started before there was any necessity for it. After the injection of 47 cc. (in 18 minutes) the abdomen was opened and the peritoneum rubbed without any reaction. About this time, however, the spontaneous respiration was shallow, lid reflex slight, and the legs were limp. In the following half hour the rate of injection was considerably reduced—about 16 cc. in 33 minutes. The loss of sensibility lasted for about half an hour longer. The spontaneous respiration returned perceptibly sooner. Altogether 63 cc. of magnesium sulphate were injected in about 51 minutes. Then, when the magnesium sulphate injection was stopped and the insufflation discontinued, the spontaneous respiration immediately appeared to be excellent. No calcium chloride was given. After placing the animal on the floor, motility and sensibility returned fairly soon. The quan-

tity of magnesium given in this experiment was not large and the rate of injection slowed down considerably during the latter part of the experiment. The recovery here was prompt and without the aid of calcium chloride.

Experiment 5.—Bull terrier, female; weight 7,600 gm.

Preparation the same as in previous experiments.

Rectal temperature 40.2°. Femoral pulse 30 to $\frac{1}{4}$, small, regular, good tension. Respiration slow, 12 to $\frac{1}{2}$, with active expiration.

10.58. Start $\frac{M}{4}$ magnesium sulphate into jugular vein.

11.04. 17 cc. Lid reflex prompt but not sustained; pupils wide. Respiration less deep, slow. Swallows occasionally.

11.06. 25 cc. 14 respirations to $\frac{1}{2}$, good depth, moderate active expiration.

11.07. 29 cc. Lid reflex prompt and sustained. Quiet.

11.08. 35 cc. Pulse small, fairly soft, 38 to $\frac{1}{4}$, regular. Respiration good depth.

11.10. 43 cc. Respiration slow, less deep but still good, 7 to $\frac{1}{4}$. Slow magnesium inflow.

11.13. 50 cc. Respiration improved.

11.15. 53 cc. Magnesium inflow slowed. Lid reflex fairly prompt.

11.18. 56 cc. Open abdomen; rub peritoneum and diaphragm; no motion of any kind. Respiration good, more rapid than before.

11.20. 58.5 cc. Lid reflex slight; respiration rapid, 36 to $\frac{1}{4}$, next count 28 to $\frac{1}{4}$; legs relaxed. Expose left sciatic nerve; no motion at first, later moderate general movements.

11.25. 71 cc. Respiration excellent.

11.29. 86 cc. Respiration much shallower and slower. Start pharyngeal insufflation.

11.32. 98 cc. No lid reflex.

11.34. 102 cc. No definite respiration.

11.37. 104.5 cc. Stimulated left intact sciatic with Petzold inductorium. At coil distances of 200 and 120 mm. no reaction elicited. At 80 mm. respirations appeared during stimulation; left toes moved slightly; also weak general motions and movements of tail. Rub peritoneum and diaphragm; no response.

11.42. 107 cc. Stop pharyngeal insufflation; slight spontaneous respiration present; start pharyngeal insufflation; increase magnesium flow slightly. Limp. No lid reflex; pupils very wide.

11.47. Abdomen and thigh wound sutured. 116 cc. No lid reflex.

11.49. 117 cc. Stop magnesium. Spontaneous respiration very slight; pharyngeal insufflation necessary.

11.50. 60 cc. $\frac{M}{4}$ sodium sulphate into jugular vein. Respiration improved promptly. Stop pharyngeal insufflation.

11.53. Pulse good. Suture neck wound, lid reflex slight.

11.56. Placed on floor, holds head up for short time, then rests it on floor; cannot stand. Wags tail when called.

12.01. Pressure on tail and toes; moves head towards tail, draws away foot.

12.05. Lid reflex very slight, pupils wide. Able to get up but prefers to squat or lie down.

1.55. Walks about readily, keeping left hind leg lifted (left sciatic nerve had been exposed), no staggering; lid reflex prompt and sustained. Pupils well contracted. Urinated large amount; first time since injection.

4.45. Walks about easily when placed on feet (staid in one place since last note); no more urine passed. Killed by chloroform.

This dog had from the start a slow respiration although its temperature was higher than normal. During the first 10 minutes of the magnesium infusion 3.5 cc. per minute were injected, more than in any of the animals in previous experiments. There were no struggles. After injecting 56 cc. the abdomen was opened and the parietal peritoneum rubbed without any reaction, while the respiration was good and even more rapid than before. After 102 cc. no definite respirations were present, and after 104 cc. the motor nerve endings were affected. The inflow was then reduced—only 13 cc. in 12 minutes. Altogether 117 cc. were injected in 51 minutes. There was practically no spontaneous respiration when the infusion of the magnesium solution was discontinued. However, the respiration improved within 1 minute after the injection of 60 cc. of $\frac{M}{4}$ sodium sulphate. The general motor and sensory depression seemed also favorably affected by this injection.

Experiment 6.—Black male; weight 7,500 gm.

Preliminary preparation as in Experiment 1.

3.13. Start $\frac{M}{4}$ magnesium sulphate into jugular vein.

3.17. 12 cc. Respiration 8 to $\frac{1}{4}$. Good depth. Femoral pulse 29 to $\frac{1}{4}$, regular, good volume and tension.

3.18. 15 cc. Lid reflex prompt and strong, pupil moderately dilated. Dog quiet.

3.20. 25 cc. Good respiration, 12 to $\frac{1}{4}$, active expiration stronger. Femoral pulse 32 to $\frac{1}{4}$, regular, good tension. Barks.

3.22. 31 cc. Lid reflex prompt and sustained; pupils wider.

3.23. 34.5 cc. Pain abolished; opening of peritoneum; respiration easy, good depth and frequency. Abdomen relaxed.

3.25. 46 cc. Rub peritoneum and diaphragm; no movement. Lid reflex prompt and sustained; respiration slow, good depth, 11 to $\frac{1}{4}$.

3.27. 50 cc. Blood bright red. Slow magnesium inflow.

3.28. 51 cc. Rub peritoneum of diaphragm; no movement. Lid reflex weak.

3.30. Pulse 30 to $\frac{1}{4}$, regular, good volume and tension.

3.31. 53.5 cc. Slight knee jerk.

3.32. 55 cc. Moved legs; respiration faster and deeper. Rub peritoneum and diaphragm; no immediate effect, after a few seconds rapid respiration with moderate strength. Increase magnesium inflow.

3.34. 61 cc. Lid reflex a mere flick; rapid respiration with active expirations in short group, then easy respirations without active expirations.

3.39. Respiration slow, good depth with active expiration.

3.42. 85 cc. Blood a little darker; respiration shallower.

3.44. Start pharyngeal insufflation.

3.46. Pulse 20 to $\frac{1}{4}$, fair volume and tension, regular. No lid reflex. Rub peritoneum and diaphragm; no movement. Legs limp; no knee jerk.

3.52. No spontaneous respiration on stopping insufflation.

3.55. Pupils well dilated but not maximal; no lid reflex. 103 cc. Pulse small, 25 to $\frac{1}{4}$.

4.00. 104.5 cc. No lid reflex. Rub peritoneum; no movement; no knee jerk.

4.05. No spontaneous respiration on stopping insufflation; pulse weak. Start insufflation again.

4.07. No movement on rubbing peritoneum and diaphragm. 106 cc. Stop magnesium. No lid reflex. Injected 60 cc. $\frac{M}{4}$ sodium sulphate into jugular vein.

4.10. Femoral pulse 25 to $\frac{1}{4}$, small, regular, better tension. Rubbing peritoneum and diaphragm; no movement.

4.11. Stop insufflation; slow respiration, getting deeper; start insufflation again. No lid reflex; pupil wide.

4.13. Rub peritoneum; no movement. Legs limp; no knee jerk.

4.17. Suture abdomen and neck wound and stop insufflation. Pulse 27 to $\frac{1}{4}$, regular, good volume and tension; 4 respirations to $\frac{1}{4}$, good and deep, no active expiration; no lid reflex, pupils wide; 36.4°. Placed on floor; cannot stand, lies on side. Pain sensation fair; looks about and wags tail.

4.25. No lid or corneal reflex; wags tail when called; feeble knee jerk; on moderate pressure of toe pads, no movement. When lifted and placed on floor front legs bear body weight, but not the hind legs. Pupils widely dilated.

4.30. Respiration easy, good depth, 14 to $\frac{1}{2}$, no active expiration. Femoral pulse 29 to $\frac{1}{4}$, regular, good volume and tension. Lies on side, wags tail. Very slight lid reflex.

4.45. Sits up on haunches, but does not walk about; lid reflex fairly good. Passed small amount of urine.

5.00. Walks about, no weakness; lid reflex prompt and sustained; pupils still wide. Killed with chloroform.

This strong dog received in the first 10 minutes about 35 cc. of the magnesium solution. There was very little excitation, and at the

end of this period the skin of the abdomen could be incised to the peritoneum without any reaction. In the following 2 minutes 11 cc. were infused, and the sensitive parts of the parietal peritoneum were energetically rubbed without producing any reaction, while respiration was still good and the lid reflex prompt and sustained. The rate of injection was now reduced, and the spontaneous respiration kept up efficiently for some time. After 19 minutes during which time about 44 cc. were injected (a little less than 2.5 cc. per minute) pharyngeal insufflation was started. In the next 23 minutes only about 16 cc. were injected (about 0.7 cc. per minute). During this period there were no spontaneous respiration, no lid reflex, no knee jerk, and the legs were limp; finally the pulse became weaker. Altogether 106 cc. of the magnesium solution were injected in 54 minutes. At the end of the magnesium injection no calcium chloride was given, but, as in the previous experiment, 60 cc. of sodium sulphate in $\frac{1}{4}$ solution were injected intravenously. The effect of the injection in this experiment, however, was in no way striking. The respiration did not improve at once and the insufflation had to be continued for about 10 minutes longer. The fact should be borne in mind that in this experiment the rate of injection of the magnesium sulphate during the first half of the infusion period was considerably greater than in any of the other experiments.

In addition to the foregoing experiments we wish to record briefly the exceptional course of one of the experiments. This dog had an irregular heart beat and its extremities were rigid before the experiment was begun. There was no spontaneous respiration after injecting 44 cc. of magnesium solution (in 14 minutes), while the peritoneum remained sensitive and the lid reflex active during most of the injection period. The animal received 72 cc. in 57 minutes. The pulse was small and often weak during the last half hour. At the end of the magnesium injection 10 cc. of $\frac{M}{8}$ calcium chloride were injected without restoring the spontaneous respirations. A few minutes later 5 cc. more of the calcium chloride brought on some weak respirations, but the heart stopped soon after and the animal died.

Here was a case in which calcium did not restore the respiration which had been abolished by magnesium; on the contrary, it was perhaps instrumental in accelerating cardiac death.

SUMMARY AND CONCLUSIONS.

These experiments justify the following general conclusions.

By the intravenous injection of $\frac{M}{4}$ magnesium sulphate into dogs at a certain rate, a stage can be reached where the abdominal walls are completely relaxed and when section of the abdomen and stimulation of sensitive parts of the parietal peritoneum do not produce pain or elicit any reaction of the animal. At the same time spontaneous respiration may still be maintained within normal limits and the lid reflex be fair or even normal. In this stage intratracheal intubation for artificial respiration can be easily accomplished. This stage may be attained in 12 to 14 minutes when the rate of injection is about 3 cc. per minute. When this stage is once attained the rate of injection should gradually be reduced, otherwise, sooner or later, spontaneous respiration will be abolished, and by a further maintenance of the rate of injection all the skeletal muscles may become paralyzed.

When the injection of magnesium is continued for a longer period, the paralytic effects of the magnesium injection will set in, even when administered at a slow rate.

The paralysis of the respiratory function is readily met by intrapharyngeal insufflation, which is easily executed even without training in this procedure, or by the method of intratracheal insufflation, if executed by one trained in its management.

When the respiration of the animal is accomplished by insufflation, the paralytic effect of the magnesium may be abolished fairly rapidly by an intravenous injection of about 10 cc. of an $\frac{M}{8}$ calcium chloride solution; or it may disappear slowly, after the infusion of the magnesium solution is discontinued for some time. The latter mode of disappearance may be favorably accelerated by an intravenous infusion of 60 to 100 cc. of an $\frac{M}{4}$ solution of sodium sulphate.

The production of anesthesia by intravenous injection of magnesium sulphate should not be undertaken unless an apparatus for intrapharyngeal insufflation is at hand, because in exceptional cases the disappearance of spontaneous respiration may be one of the earliest consequences of the magnesium injection.

The injection of calcium chloride should not be employed in cases in which the subject shows cardiac insufficiency. In such instances, moreover, injections of magnesium should not be used for the purpose of anesthesia; at least not until greater experience has been acquired in the employment of this method.

AN EXPERIMENTAL STUDY OF THE ADDITIVE AND ANTAGONISTIC ACTIONS OF SODIUM OXALATE, AND SALTS OF MAGNESIUM AND CALCIUM IN THE RABBIT.

By F. L. GATES, M.D., and S. J. MELTZER, M.D.

(*From the Department of Physiology and Pharmacology of the Rockefeller Institute for Medical Research.*)

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INTRODUCTION

On the basis of the hypothesis that magnesium favors inhibition of the various functions of the nervous system, Meltzer and Auer studied extensively in this laboratory the action of magnesium salts upon various animals. In injecting magnesium sulphate subcutaneously,[1] they found that a certain dose, which varies with the species of animals, is capable of producing profound anesthesia and paralysis from which the animal recovers. For rabbits this dose amounts to about 1.5 gm. of magnesium sulphate ($MgSO_4 + 7\ H_2O$) administered in a molecular solution. Larger doses cause the death of the animal, as a rule, by respiratory paralysis. With an effective but non-fatal dose in subcutaneous injections the development of the depressing, inhibitory effect is gradual and fairly slow. When the maximum is reached, the turn for the recovery sets in soon; there is practically no real plateau to the inhibitory curve. The descending limb of this curve—the recovery—is steeper than the ascending one. When a magnesium salt is injected intramuscularly, the inhibitory as well as the fatal effects set in more promptly and with smaller doses.

In the course of their studies, Meltzer and Auer[2] found that calcium, which is chemically closely related to magnesium, is biologically appar-

[1] Meltzer, S. J., and Auer, J., *Am. Jour. Physiol.*, 1905, xiv, 366.

[2] Meltzer, S. J., and Auer, J., *Am. Jour. Physiol.*, 1908, xxi, 400.

ently the antagonist of the latter. When calcium is injected intravenously, shortly before or immediately after the respiration stops, into an animal which has received a fatal dose of magnesium, the animal will recover in less than a minute, provided, of course, that the circulation is still effective during the calcium injection. This biological antagonism is a remarkable fact and can be made the basis of many problems worth investigating. So far, at least, it is not known that between calcium and magnesium salts which have the same anion a chemical antagonism exists; no precipitation, for instance, occurs *in vitro* when a solution of magnesium chloride is mixed with a solution of calcium chloride. Calcium chloride is nevertheless strikingly antagonistic to magnesium chloride as far as the life of animals and plants is concerned.

What effect would the deprivation of the animal body of some of its calcium have upon the behavior of the animal? There are a number of acids and salts which precipitate calcium compounds *in vitro*. Will the administration of these calcium-precipitating compounds, let us say oxalic acid or oxalates in general, bring out symptoms indicating an increase of magnesium action? By precipitating calcium within the body a certain amount of unantagonized magnesium would be set free. Would this fact become manifest by the appearance of inhibitory and paralytic phenomena? The symptoms of oxalate poisoning do not speak for it; in general they possess rather the opposite character: excitation, tremor, and convulsions. But the amount of magnesium thus set free and the inhibition which it may be capable of exerting, might under these circumstances be too small to play a perceptible part, in the presence of the violent opposite symptoms which are brought out by another exciting factor of the oxalate. Could, however, the depressing component of the calcium-precipitating oxalate be brought out by a simultaneous administration of a subminimal dose of a magnesium salt? This was the problem which we tried to solve experimentally.

While we were at work on this problem, Schütz[3] published a brief preliminary communication in which he says that the susceptibility to magnesium injections could be increased occasionally, but not

[3] Schütz, J., *Wien. klin. Wchnschr.*, 1913, xxvi, 745.

constantly by sodium oxalate. A few months later Starkenstein,[4] with whose work we were not familiar until after we had given a preliminary communication of our results,[5] stated in a preliminary report that he found "like Schütz that the addition of oxalates constantly gave a visible increase of the magnesium narcosis."

We shall describe briefly our experiments bearing upon the problem under discussion and the conclusions to which they point.

EXPERIMENTAL PART.

We experimented exclusively on rabbits. Magnesium sulphate in M solution ($MgSO_4 + 7\ H_2O$) and sodium oxalate in 3 per cent solution[6] were injected separately and practically simultaneously, either into the lumbar muscles on opposite sides of the spine, or subcutaneously into each flank, the injection being usually followed by brief massage. All doses were estimated and are here reported in gm. of the salt per kilo of body weight. Most of the experiments were performed on a series of three animals, two serving as controls and receiving subtoxic doses of either magnesium sulphate or sodium oxalate alone. The experimental animal received the same dose of both salts.

Intramuscular Injections.

An abbreviated typical protocol follows.

Experiment I.

Rabbit A.—Oct. 2, 1913. Magnesium sulphate alone. Grey female. Weight 1,550 gm.

11.10 Right lumbar muscles: magnesium sulphate M, 4.3 cc. = 0.7 gm. per kilo of body weight.

11.23. Lying down, head up, breathing rapidly.

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[4] Starkenstein, E., *Wien. klin. Wchnschr.*, 1913, xxvi, 1235.

[5] Gates, F. L., and Meltzer, S. J., *Zentralbl. f. Physiol.*, 1913–14, xxvii, 1169. Starkenstein, E., *Zentralbl. f. Physiol.*, 1914, xxviii, 63; *Arch. f. exper. Path. u. Pharm.*, 1914, lxxvii, 45.

[6] Merck's reagent, "Sörensen's oxalate." Impure oxalates are not soluble to 3 per cent.

11.32. Can be placed on side.

11.34. Moves eyes and head when approached.

11.40. When tail is pressed, raises head and turns on belly. Moves head and looks around.

11.57. Hops when tail is pressed.

12.13. Hops around voluntarily, watching other rabbits. Remains well.

Rabbit B.—Oct. 2, 1913. Magnesium sulphate and sodium oxalate. Grey male. Weight 1,720 gm.

11.13. Right lumbar muscles: magnesium sulphate M, 4.8 cc. = 0.7 gm. per kilo. Left lumbar muscles: sodium oxalate 3 per cent, 5.6 cc. = 0.10 gm. per kilo.

11.24. Lying prone, with head on floor. When pushed over on side, lies passive. Respiration full; 17 in ¼ min. No response to pressure on tail; lid reflex good.

11.38. Same position. Lid reflex hardly perceptible.

12.01. Respiration slow and deep; 13 in ¼ min.

12.12. No response to stimuli, no lid reflex.

12.37. Turns over and lies on belly. Head sinks to floor and is raised at intervals. No response to pressure on tail.

1.08. Crouching in a corner. Recovers.

Rabbit C.—Oct. 2, 1913. Sodium oxalate alone. Grey male. Weight 1,480 gm.

11.21. Left lumbar muscles: sodium oxalate 3 per cent, 4.88 cc. = 0.1 gm. per kilo of body weight.

11.28. Sitting up, alert, changes position frequently. Starts suddenly without apparent cause.

11.43. Active, hops around, laps water, licks site of injection.

12.14. Has been behaving normally. Does not remain in one position long. No further effects noted.

The contrast in the behavior of the controls and the experimental animal is striking. The control animals were but little affected: this dose of sodium oxalate produced only trivial symptoms in Rabbit C, and the magnesium animal, Rabbit A, while weak and stupid, was at no time paralyzed or anesthetic. The third rabbit, however, ten minutes after the injections, was deeply anesthetized and remained passive and insensible for an hour. Two points are of particular interest: (1) In spite of the depth and long duration of the narcosis the respiration continued of good volume and rate and the animal was at no time in danger. (2) The animal regained power of voluntary movement before the return of sensibility to painful stimuli. A series of experiments with similar doses is given in Table I.

TABLE I.

Magnesium Sulphate and Sodium Oxalate, Intramuscularly.

No. of experiments.	Dose per kilo of body weight.		Average duration of deep inhibition. Animal relaxed on side.	Died.	Remarks.
	Magnesium sulphate.	Sodium oxalate.			
	gm.	*gm.*	*min.*		
6	0.7	0.10	89	0	
6	0.7	—	10	0	Only two out of six relaxed at all.
4	—	0.10	0	0	Visible effect questionable.

Subcutaneous Injections.

When the injections were made subcutaneously, somewhat larger doses had to be employed. A typical protocol of an experiment follows.

Experiment II.

Rabbit A.—Oct. 9, 1913. Magnesium sulphate alone. Slate colored female. Weight 2,030 gm.

10.16. Left flank, subcutaneously: magnesium sulphate M, 6.5 cc. = 0.8 gm. per kilo. Massage for 20 seconds.

10.31. Lying down, head and ears erect, breathing rapidly.

10.47. When disturbed hops away clumsily.

11.15. Crouching quietly, head up, ears flat on back. Respiration good.

11.50. Raises head to sniff at nearby objects.

12.20. Sitting up, washing paws. Remains well.

Rabbit B.—Oct. 9, 1913. Magnesium sulphate and sodium oxalate. Grey male. Weight 1,755 gm.

10.18. Right flank, subcutaneously: magnesium sulphate M, 5.6 = 0.8 gm. per kilo. Left flank, subcutaneously: sodium oxalate 3 per cent, 8.75 cc. = 0.15 gm. per kilo. Both sides massaged for 20 seconds.

10.53. Sitting up naturally.

11.04. Lying with chin on floor. Respiration slower and deep; 21 in ¼ min.

11.13. Placed passively on side without a struggle. Respiration 18 in ¼ min.

11.37. No response to pressing tail. Respiration shallow.

12.50. Trace of lid reflex. No response to pressing tail. Respiration of fair depth; 14 in ¼ min.

2.35. No lid reflex. No response to pressing tail.

3.52. Animal lying as before. No response to stimuli. Breathing entirely abdominal, of fair depth; 14 in ¼ min.

4.28. Does not resist handling. Voluntarily moves head, tail, and legs slightly. Observation discontinued.

Oct. 10, 1913. 9.15. Sitting up in cage. Rather quiet.

Rabbit C.—Oct. 9, 1913. Sodium oxalate alone. Grey female. Weight 1,510 gm.

10.22. Left flank, subcutaneously: sodium oxalate 3 per cent, 7.5 cc. = 0.15 gm. per kilo. Massage for 20 seconds.

10.43. Hops around licking the floor and sniffing at objects.

10.53. Sitting up, behaving normally.

11.14. Hops off actively when approached. No effects noted from injection.

Here again neither the oxalate nor the magnesium alone was effective. Together they produced a profound depression with a period of anesthesia and paralysis lasting more than four hours, followed by a gradual complete recovery.

Table II summarizes experiments with subcutaneous injections.

TABLE II.

Magnesium Sulphate and Sodium Oxalate, Subcutaneously.

No. of experiments.	Dose per kilo of body weight.		Average duration of deep inhibition. Animal relaxed on side.	Died.	Remarks.
	Magnesium sulphate.	Sodium oxalate.			
	gm.	*gm.*	*min.*		
8	0.8	0.15	123 +++	1	In four animals anesthesia extended into the night following. One died next day without recovery.
8	0.8	—	0	0	Practically no effect. Drowsiness in four cases.
8	—	0.15	0	0	No effects observable.

The cited protocols and the two tables illustrate the results obtained in these series of experiments. With the exception of two failures at the beginning, before the proper relation of dosage was determined, the experimental animal in every instance was definitely more deeply affected than the controls. The differences between the various experiments were only of degree, and depended upon the relation of the dose employed and the mode of administration, whether subcutaneous or intramuscular. With proper dose the

contrasts were striking and constant; while the controls were hardly visibly affected, the experimental animals were deeply anesthetized and paralyzed, the character of this inhibition being in general similar to that caused by large effective doses of magnesium alone.

In the following particulars the depression of the animals which received sodium oxalate and magnesium sulphate seemed to differ from that of animals which received magnesium alone. (1) The period of anesthesia and paralysis is a fairly long one, especially after subcutaneous injections, when the state of inhibition may last even 4 hours and longer; whereas after an effective sublethal dose of magnesium alone the entire state of depression is of a comparatively short duration. (2) In animals which receive oxalate and magnesium the deepest stage of anesthesia and paralysis tends to become stationary and is of long duration—the inhibitory curve has a long plateau—and the recovery takes place gradually; whereas with magnesium alone the inhibitory curve has hardly any plateau, and the animal after reaching the acme of anesthesia and paralysis either recovers quite rapidly or the depression leads to death by respiratory paralysis.

The increase of depression following the injection of subminimal doses of sodium oxalate and magnesium sulphate which was definitely established in these experiments cannot be considered simply as a summation of two similar effects. The symptoms brought on by oxalates are entirely dissimilar to those of magnesium inhibition. In our experiments the symptoms which follow the injections of sodium oxalate in toxic doses exhibit the character of excitation; anxiety, restlessness, hypersensitiveness, and tonic and clonic convulsions, which finally lead up to asphyxia and to a fatal termination. In such subminimal doses as we have employed, the toxic symptoms, if there were any, consisted at most in excitation and increased alertness; but there was never any manifest depression. It seems, therefore, that the strikingly depressing effect which the addition of a practically non-toxic dose of sodium oxalate to a subminimal dose of magnesium produces, must be ascribed to the ability of the oxalate to precipitate calcium from the body fluids and thus eliminate an element which biologically is antagonistic to magnesium.

The Action upon the Motor Nerve Endings.

Among the general effects of magnesium salts their depressing action upon the motor nerve endings stands out prominently. In minimal effective doses these salts reduce and in larger doses they completely abolish the conductivity of the nerve endings. In a series of experiments we have studied directly the combined action of sodium oxalate and magnesium sulphate upon this intermediary link between nerve and muscle. The sciatic nerve was cut under ether, the animal permitted to recover completely, and then the motor reactions of foot and toes to faradic stimulations of the peripheral end of the sciatic nerve were studied under the influence of the salts under discussion. Seventeen experiments were made upon rabbits. In fifteen there were two rabbits to each experiment, one an experimental animal and one a control. The experimental animals received subcutaneous injections of 0.6 to 0.8 gm. of magnesium sulphate in one side and 0.15 to 0.2 gm. of sodium oxalate. The fifteen control animals received injections of 0.6 to 0.8 gm. of magnesium sulphate alone. Two rabbits received injections of 0.15 and 0.2 gm. of sodium oxalate alone. For faradic stimulations a Porter induction coil, armed with one Daniell cell, was used. The cut sciatic nerve was stimulated before and at various intervals after the injection of the salt solutions, and the degree of the reactions to the various strengths of stimuli was noted. The results obtained in the experimental and control animals were compared and brought into relation with the general condition of the respective animals.

In both the sodium oxalate animals stimulation of the sciatic nerve before and at various times after the injection gave prompt reactions; strong tetanic flexion of the foot and abduction of the toes.

In eight of the magnesium controls stimulation of the sciatic nerve gave normal responses at the various periods after the injection. In the seven other controls there were slight degrees of reduction in the response to the stimulations; the reaction was less prompt, the extent of the contractions was lessened, or the distance of the secondary coil, in order to be effective, had to be shortened.

Of the experimental animals, in thirteen the conductivity of the peripheral nerve endings was definitely more deeply affected than in

their controls. In some cases the conductivity was so depressed that at the time when the general narcosis was at its height no response could be obtained from the stimulation of the sciatic nerve even with a 40 mm. coil distance. In two of the experimental animals the reduction in the response to stimulation of the sciatic nerve was not greater than that of their controls, although the general signs of anesthesia in the experimental animal were quite deep.

The depressing effect upon the motor nerve endings never outlasted the central effects, while there were cases in which the loss of sensation still continued after the motility seemed to be normal again.

The Antagonistic Action of Calcium.

Calcium, as stated in the introduction, is biologically antagonistic to magnesium, and our present experimental results led us to the conclusion that the increase of the depressive action of subminimal doses of magnesium by the addition of a subtoxic dose of sodium oxalate was due to the calcium-precipitating property of this salt. On the other hand, we found that the anesthesia and paralysis produced by a combination of subminimal doses of the two salts was of much longer duration than the same condition produced by an effective dose of magnesium sulphate alone. The question presented itself: Would calcium cause a recovery from the profound long-lasting state of depression caused by the combined action of the two salts, and especially would the recovery be as prompt and as rapid as in cases of magnesium anesthesia? We made a large number of experiments, but our results may be presented in the following single sentence: The antagonistic action of calcium is just as striking and prompt in the prolonged anesthesia brought about by the combination of oxalate and magnesium as it is in the anesthesia produced by magnesium alone. The following protocol is typical for all experiments in this series, and the photographs (Figs. 1 and 2) taken of this experiment are a good illustration of the results.

Experiment III.

Rabbit I.—Mar. 9, 1914. Magnesium sulphate alone. Grey and white male. Weight 1,860 gm.

1.58. Right back, subcutaneously: magnesium sulphate M, 5.9 cc. = 0.8 gm. per kilo. Massage for 1 min.

2.16. Sits quietly in corner of box, or lies down.

2.23. Crouching on forepaws, head and ears up. Respiration fair volume, slow; 19 in ¼ min.

2.32. Lying at full length, head and ears up. Backs up into sitting posture; rather heavy and quiet.

2.47. Crouching quietly in corner of box. Respiration full volume; 14 in ¼ min.

3.07. Photographed (Fig. 1).

3.16. Photographed (Fig. 2).

3.45. Behaving normally and has shown no further effects. Remains well.

Rabbit II.—Mar. 9, 1914. Magnesium sulphate and sodium oxalate. Black and white female. Weight 1,540 gm.

2.01. Right back, subcutaneously: magnesium sulphate M, 4.9 cc. = 0.8 gm. per kilo.

2.03. Left back, subcutaneously: sodium oxalate 3 per cent, 7.7 cc. = 0.15 gm. per kilo. Massage both sides for 1 min.

2.10. Has defecated. Respiration rapid and rather deep. Restless, changes position often.

2.13. Hind legs dragged a little in walking.

2.21. Crouching, head up, ears back, breathing rapidly; 68 in ¼ min.

2.35. Lying full length, eyes half closed, ears back, chin on floor. Flanks relaxed and bulging. Respiration 50 in ¼ min.

2.49. Lying partly on side, relaxed, head flat on floor. Mere trace of lid reflex. Moves head slightly when tail is touched.

3.05. Placed passively on back, feet in air. Remains there relaxed.

3.07. Photographed with controls (Fig. 1).

3.15. Same condition. Given 8 cc. calcium chloride 0.125 M through left ear vein. Respiration deepens during injection, and before it is completed animal turns over and sits up.

3.16. Photograph taken within 1 minute of injection (Fig. 2).

3.45. Crouching quietly. Hair erect. Hops off actively when disturbed. Then sits up with head and ears up. Remains well.

Rabbit III.—Mar. 9, 1914. Sodium oxalate alone. White female. Weight 1,620 gm.

2.06. Left back, subcutaneously: sodium oxalate 3 per cent, 8.1 cc. = 0.15 gm. per kilo. Massage for 1 min.

2.11. Hind legs dragged a little at times. Rather restless.

2.30. Sitting up or hopping around naturally. Head and ears up. Not restless or anxious. Respiration 42 in ¼ min.

2.48. Behaving normally. Sitting up, quiet. Respiration 35 in ¼ min.

3.07. Photographed (Fig. 1).

3.16. Photographed (Fig. 2).

3.45. Has shown no further effects.

Figs. 1 and 2 illustrate, in the first place, the anesthesia and paralysis produced by the combination of subminimal doses of magnesium and oxalate. They show, further, in a striking way, the antagonistic action of intravenous injection of calcium; it is in all respects similar to the action of calcium in anesthesia by magnesium alone. The respiration becomes deeper and more rapid immediately after beginning the injection, and the return of muscle tone and motor activity can be felt under the hand. Within a minute after the beginning of the injection, often indeed before all of the 8 or 10 cc. of solution is given, the animal draws up its legs, raises its head, turns over and scrambles into a sitting posture, and becomes alert and inquisitive. After an interval the rabbit may gradually sink back into narcosis, and can be restored again by calcium. Occasionally, if too much magnesium and oxalate have been given, a third injection may still be needed and given with success. However, under such circumstances, repeated injections of calcium might finally prove fatal to the animal.

The experiments, showing the depressing effect of magnesium and the antagonistic action of calcium to this depression, are, as we had occasion to learn, frequently demonstrated in many European Universities in lectures on pharmacology or physiology. When magnesium alone is used, the period of the greatest depression is of short duration and the demonstration may either be unconvincing, when the animal is not yet sufficiently narcotized, or it may be a failure, when the calcium injected is administered too late. The anesthesia and paralysis brought about by a combination of sodium oxalate and magnesium sulphate is, as we have seen above, of comparatively long duration. It is therefore a more appropriate method for purposes of demonstration. The animal may receive its double injection 40 to 50 minutes before the time set for the demonstration. If the proper doses are given and the proper procedure is followed out, there is no danger that the animal will not be in deep anesthesia, or that it will die too soon, before the antagonistic effect of the calcium can be shown.

SUMMARY.

The foregoing experiments establish firmly the following facts.

Subcutaneous or intramuscular injections of sodium oxalate in subtoxic doses, when administered to an animal which received a subminimal dose of magnesium sulphate, produce profound anesthesia and paralysis of long duration, although the usual effects of sodium oxalate alone are of a stimulating character. This fact is, in general, in harmony with the results reported by Starkenstein who, however, seems to have used the combination of the two salts in one solution; namely, that of magnesium oxalate.

The combined injections of subminimal doses of sodium oxalate and magnesium sulphate produce a strong reduction, or even, at times, a complete abolition of the conductivity of the motor nerve endings.

An intravenous injection of calcium salts brings on a recovery from the profound and prolonged effects of the combined action of sodium oxalate and magnesium sulphate, which is as prompt as is observed in experiments in which effective doses of magnesium alone were given. This fact is the more noteworthy, since depressions of long duration produced by prolonged continuous injections of magnesium solutions alone do not respond very promptly and effectively to calcium injections.

As will be recalled, the starting point for our investigation was the hypothesis that substances which are capable of precipitating calcium —a biological antagonist of magnesium—ought to be capable of increasing the depressive effect of magnesium. Our experiments proved that this assumption was correct. This would seem, therefore, to justify the interpretation that the augmenting action of sodium oxalate has its cause in the ability of the latter to precipitate calcium and thus increase within the body the amount of unantagonized magnesium. However, we wish to state expressly that this view is, for the present, still no more than a hypothesis and does not exclude other possible interpretations of our facts. As we pointed out it speaks against this hypothesis that oxalates do not produce phenomena of depression; the toxic symptoms produced by oxalates exhibit distinctly signs of increased and not of decreased irritability.

EXPLANATION OF PLATE 95.

FIG. 1. Rabbit III. Sodium oxalate 3 per cent, 0.15 gm. per kilo. Alert, ears erect. (Caught by instantaneous exposure.) Rabbit II. Sodium oxalate 3 per cent, 0.15 gm. per kilo. Magnesium sulphate M, 0.8 gm. per kilo. Deeply anesthetized and quite relaxed. Rabbit I. Magnesium sulphate M, 0.8 gm. per kilo. Crouches quietly as placed. Ears back.

FIG. 2. Rabbits III and I as before. Rabbit II within a minute has received 8 cc. of calcium chloride 0.125 M into the marginal ear vein (note clip). Alert and sensitive; right paw blurred from movement.

III II I

FIG. 1.

III II I

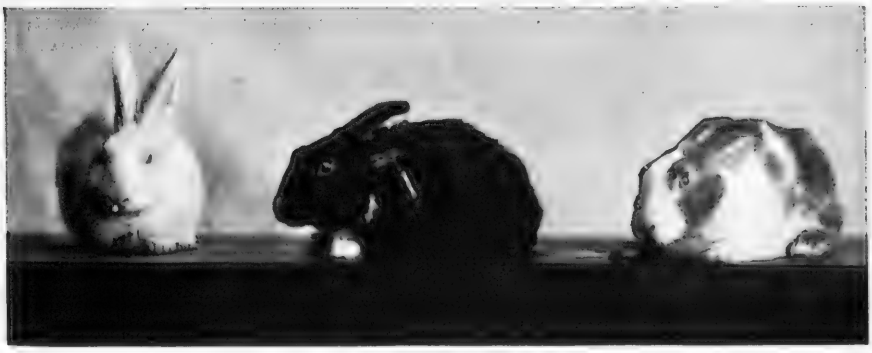

FIG. 2.

(Gates and Meltzer: Action of Sodium Oxalate, Magnesium, and Calcium Salts.)

THE DISTRIBUTION OF TRYPAN-RED TO THE TISSUES AND VESSELS OF THE EYE AS INFLUENCED BY CONGESTION AND EARLY INFLAMMATION.

By PAUL A. LEWIS, M.D.

(*From the Henry Phipps Institute of the University of Pennsylvania, Philadelphia.*)

(Received for publication, February 24, 1916.)

It is the purpose of this paper to present certain simple observations which have a bearing on the manner in which the constituents of the aqueous humor are secreted into and eliminated from the anterior chamber of the eye. It will be shown that external influences may affect profoundly the ease with which abnormal constituents appear in the anterior chamber fluid. The observations have some bearing on general problems of pathology and pharmacology which will be pointed out. And lastly there will be included some observations on the diffusion of substances into and from the cornea.

All the observations have been made on the rabbit's eye. The azo-dye trypan-red as furnished by Grübler has been the substance chiefly employed for testing the permeability. This dye has been dissolved in normal saline solution to the amount of one-quarter of 1 per cent. 100 cc. of this solution may be warmed to body temperature and injected slowly into a full grown rabbit by the intravenous method without causing the animal any immediate distress and with no appreciable evidence of toxicity later. Under these circumstances by the time the injection is finished the skin and mucous membranes of the entire body are stained red. The intensity of the stain in the tissues increases for a number of hours to a maximum which is maintained without appreciable change for a number of days, and which then gradually fades out over a period of weeks and months. Trypan-blue and other azo-dyes of similar physiological activity may be used in the same general way.

Trypan-red is a colloidal substance; that is, its watery solutions do not diffuse through parchment paper. When, therefore, the dye leaves the blood vessels to appear in the tissues, the lymphatic spaces, lachrymal secretions, or the urine, its passage is in itself evidence that the interposed tissue surfaces are not perfect dialyzing membranes. They are either leaky mechanically to substances of certain physical constitution, or they exert a selective action which permits some colloids to pass while retaining others.

If the normal eyes are observed at any time after the injection of the dye in the manner described it will be seen that the sclera shares the stain of the skin with greater or less intensity. No stain can be detected in the cornea or anterior chamber fluid by inspection of the eye. If, however, after a number of hours the aqueous humor be withdrawn it will be found to have a barely appreciable pink color. If at the end of a week or 10 days the animal is killed, the eye removed, and the cornea dissected free, it also will be seen to be stained very faintly although definitely. If the blood be withdrawn immediately after the injection, the serum is found intensely stained with the dye. As the tissue stain increases in intensity, the stain disappears gradually from the blood to a minimum point which is probably long maintained.

Observations on the Aqueous Humor.

If within a few minutes after finishing the intravenous injection one eye is cocainized and the anterior chamber fluid withdrawn, a colorless fluid is obtained. As the chamber refills, which it does in the course of a short time, the reformed fluid is stained intensely. The rapidity with which a stained fluid appears in the anterior chamber following such a tap varies considerably in different rabbits. It is also possible so to alter the physiological condition of the eye that the dye when injected intravenously will quickly appear in the anterior chamber without the preliminary tapping.

With the facts above outlined as a basis for work, experiments have been carried out which throw some light on two distinct questions; namely, the place from which certain abnormal constituents may be secreted into the anterior chamber of the eye, and secondly the

relation between the different constituents of an altered anterior chamber fluid in the time of their appearance.

There is now a general agreement that the normal anterior chamber fluid is furnished from the vascularized ciliary body behind the iris, reaching the anterior chamber through the pupil. No experiment entirely free from objection has ever been devised either to prove or disprove this. The experiments in proof of the proposition have involved the withdrawal of fluid from the eye, a procedure which at once disturbs the relationships to an important degree. The experiments of Ehrlich[1] with fluorescin were interpreted by him to signify that the anterior chamber fluid was secreted from particular regions of the anterior surface of the iris. Ehrlich's experiments, done many years ago in part, show that fluorescin appears in the anterior chamber fluid from regions anterior to the iris. It should be recognized, however, that in interpreting such an experiment the fluid and the various other elements of either a normal or an altered aqueous humor may originate in different places. Those portions of Ehrlich's experiments which seemed to him to show that there were definite currents in the aqueous humor flowing from the sides toward the center, meeting on a vertical line in the midregion of the chamber in such a way as to form a swirl where the two streams meet, are susceptible of no explanation in the light of the conditions, as displayed by our work with trypan-red.

When the aqueous humor is withdrawn the pupil usually contracts more or less. Almost immediately the chamber begins to fill again with fluid. When the tap has followed shortly after the intravenous injection of trypan-red, the reformed fluid, as has been said before, is colored with the dye. The color comes for the most part through the pupil from behind. Occasionally it can be seen that the dye makes its appearance in considerable concentration on the anterior surface of the iris away from the pupilary margin before any color has appeared through the pupil.

If eserine is dropped in one eye in sufficient quantity to contract the pupil as far as possible, and if then the dye is injected intravenously, even though no aqueous humor is withdrawn the fluid will

[1] Ehrlich, P., Ueber provocirte Fluorescenzerscheinungen am Auge, *Deutsch. med. Wchnschr.*, 1882, viii, 21.

frequently become colored. Here again the color always appears from behind the pupil, but it may also appear on the anterior surface of the iris independently.

Neither cocaine nor atropine causes the appearance of color in the untapped eye. Atropine when used in such a way as to dilate the pupil fully before the eye is tapped prevents any appearance of dye on the anterior surface of the iris.

If one eye of a rabbit is cocainized and the cornea inoculated with a living culture of the tubercle bacillus, a progressive lesion results, differing in character with the particular culture used and the amount inoculated. The characteristics of this lesion we have described in detail elsewhere.[2] 24 hours after the inoculation there is usually an intense congestion of the conjunctiva, the iris and the ciliary body being more or less congested. This congestion tends to subside by the 2nd day but does not, as a rule, entirely disappear.

24 hours or more after such an inoculation, if the animal is injected with the dye intravenously as above described, the anterior chamber fluid of the inoculated, untapped eye will always become colored. Here again most of the color appears through the pupil, but in many instances it also appears earlier and independently on the anterior surface of the iris.

In each of these instances, the tapped eye, eserinated eye, or the tubercular eye, whenever the color appears on the anterior surface of the iris, it seems to be associated with definite areas of congestion. It would be interesting to know whether it is impossible to have the color appear abnormally in the absence of such a congestion. We have, with this in mind made some experiments with abrin. This poison, as is well known, causes an intense inflammation of the conjunctiva when it is dropped in the eye. The inflammation is characterized by a well marked edema and congestion and develops slowly through a stage which is, generally speaking, one of edema, to a stage which is more predominantly congestive. In the various stages of this inflammation trypan-red or trypan-blue applied intravenously appears in the anterior chamber, and in the edematous conjunctiva with unusual rapidity. We have never had a result, however, which

[2] Lewis, P. A., and Montgomery, C. M., *Jour. Exper. Med.*, 1914, xx, 269.

enables us to think that the dye can appear in abnormal amount or situation as a consequence of an edematous condition alone and in the entire absence of congestion.

To sum up this portion of our remarks we may say that under conditions in which the eye is slightly congested certain dyes injected intravenously may appear in abnormally large amount and with unusual rapidity in the aqueous humor. The largest amount of the dye comes into the aqueous humor from behind the iris, but appreciable amounts frequently come from the anterior surface of the iris. In these instances the iris has always shown a local congestion in the region in which the dye has appeared.

It is a well known fact that if the aqueous humor be withdrawn the fluid which refills the anterior chamber differs from that first withdrawn in that it coagulates spontaneously. We have tested the relationship between the appearance in the fluid of the factors controlling the coagulation and the dye. When the dye is injected intravenously, the eye being tapped at once after the injection is finished, the rapidity with which the dye appears in the anterior chamber varies greatly in different rabbits. It is usually from 20 to 30 minutes before fluid is obtained which would be classified as intensely stained. At this time the fluid has always coagulated spontaneously in our experience. Occasionally we have had animals in which at the end of 5 minutes the anterior chamber fluid was intensely stained. On withdrawing this at once we have several times obtained an intensely colored fluid which did not coagulate. These facts suffice to show that the appearance of the dye is independent of the appearance of at least some of the factors determining coagulation.

The distribution of the dye when it comes into the anterior chamber is interesting in that it throws some light on the rapidity of movement of the aqueous humor. In those instances where the dye appears in concentration on the iris surface before it comes through the pupil it diffuses very slowly from the point where it makes its appearance, and this diffusion is apt to be more or less even in all directions. When the dye comes through the pupil it is apt to come over the lower pupilary margin in a concentrated stream which sinks slowly to the bottom of the chamber and from there, spreads in the course of half an hour or more by diffusion until the aqueous is evenly colored.

The conditions can easily be duplicated outside the body. If one takes a thin collodion sac about an inch in diameter, fills it with a 1 per cent solution of trypan-red, and gently lowers it into a beaker of water the dye will diffuse out of the sac into the water with moderate rapidity. The dye comes out of the sac over the entire submerged surface. It then apparently creeps along the surface of the sac to the lower end of it and falls from there in a narrow stream of concentrated dye to the bottom of the beaker. It spreads along the bottom to make a concentrated red layer in this region. Then in the course of an hour or more it diffuses throughout the water. If the beaker is disturbed, of course the secondary spread is hastened.

The conditions in the anterior chamber of the eye are analogous to this, and it is hard to reconcile the observations with the view that there are any very active movements in the aqueous humor. There seems to be a tendency for matter coming into the anterior chamber through the pupil, to come in at the lower margin and to sink to the bottom of the chamber.

There is also a tendency for the dyes that we have used, at least, to leave the chamber in a definite region. If 0.5 cc. of a 1 per cent solution of trypan-red or trypan-blue is taken in a suitable syringe and the aqueous humor from a normal eye is also drawn into the same syringe and if then the mixed fluid and dye solution is immediately reinjected, care being taken to restore approximately the original tension, the absorption of dye can be watched very well. Within a few minutes vessels in the sclerotic will be seen to be injected with the dye. The vessels which are first injected and the only ones which usually show a pronounced injection are on the upper surface of the eyeball to each side of the midline. The injection of vessels in this region persists until the dye is completely absorbed.

The indication in these experiments is, then, that there is very little movement in the aqueous humor. The points of secretion and absorption are such as to determine fairly definite lines of diffusion for colloidal matter in the fluid, which in a general way are from behind the iris, through the pupil to the lower portion of the chamber, and then upward to leave at the superior portions of the angle.

Observations on the Cornea.

In a preceding paragraph we said that a number of days after an intravenous injection of trypan-red the cornea becomes distinctly colored. It is the generally accepted view that substances which reach the cornea do so by diffusion from the corneoscleral margin. The way in which the cornea becomes stained is in accord with this. If an animal is killed 2 or 3 days after the dye is injected, the cornea will, on examination, be found stained at its circumference, the colored area at this time reaching about one-third of the way to the center.

In connection with the study of experimental tuberculosis previously referred to, we observed that the reactions of the cornea were not uniform throughout. If, for example, a central inoculation is made, the first formation of blood vessels at the corneoscleral margin will be above, at approximately the midline. Next, vessels will form on the midline below, and, lastly, on the sides. We assumed that this was because substances diffused out of the cornea by preference toward the upper portion and hence stimulated the tissue reactions first at that point. That the diffusion is along these lines we have now found can be shown to be the case with the dyes we have used in this work. If the cornea is infiltrated in a spot 2 to 3 mm. in diameter at its center, the dye does not diffuse toward the lower corneoscleral margin in any appreciable degree. The diffusion is chiefly toward the upper margin, spreading out more or less in the shape of a fan in this direction. To get a diffusion chiefly toward the sides or lower margin it is necessary to place the infiltration quite close to the corneoscleral junction in those directions.

SUMMARY.

In as far as the observations reported have a bearing on the movements of fluid within the eye, they are, for the most part, in accord with views at present generally accepted. On the other hand, we know of no other way in which it may be so readily demonstrated that simple and even temporary local circulatory changes may profoundly alter the distribution of substances from the circulating

blood to the extravascular fluids and tissues. In the light of these observations, it would seem that such changes might easily account for marked idiosyncrasies in the action of poisonous drugs, and as well probably for other factors in drug action.

THE APPEARANCE OF THE PRESSOR SUBSTANCE IN THE FETAL HYPOPHYSIS.

By DEAN LEWIS, M.D.

(*From the Hull Laboratory of Anatomy, University of Chicago, Chicago.*)

PLATES 96 AND 97.

(Received for publication, February 21, 1916.)

It has been demonstrated (Lewis, Miller, and Matthews[1]) that the pressor substance of the posterior lobe of the hypophysis is secreted by the pars intermedia, a derivative of the pharyngeal pouch. The present study was undertaken with the hope of determining at what period in fetal life the pressor substance appears and of correlating the cytological changes in the pars intermedia with the establishment of secretory function, using the appearance of the pressor substance as an index. The cytological studies are now in progress.

I have used extracts from the entire gland. The hypophyses of pigs just before birth are large enough to permit of separation of the two lobes, but in the earlier stages this is impossible. In order to secure uniform material for injection the extracts were made from the entire gland in all instances. The glands were obtained fresh and extracted in absolute alcohol to remove the depressor substances. After extraction was completed, the alcohol was filtered off and the residue dried in a desiccator. The dried residue was kept in small bottles until desired for use, when a salt solution extract was made for intravenous injection.

Fetal pigs just before birth measure from 275 to 285 mm.; the measurement being taken from the crown of the head to the tip of the coccyx. Fig. 1 indicates the effect upon blood pressure of an intravenous injection of 0.1294 gm. of dried hypophyses removed from pigs just before birth, extracted in 5 cc. of normal salt solution.

[1] Lewis, D., Miller J. L., and Matthews, S. A., The Effects on Blood-Pressure of Intravenous Injections of Extracts of the Various Anatomical Components of the Hypophysis, *Arch. Int. Med.*, 1911, vii, 785.

This extract must be carefully filtered before injection, for much of the dried glands is insoluble. Intravenous injection of 5 cc. of this extract caused a prompt and marked rise in blood pressure which was followed by a fall and a second rise to almost the same level as the first, accompanied by marked slowing of the pulse and an increase in the length of the pulse wave. This type of tracing is often obtained after intravenous injections of extracts made from adult glands, but it is not the most common type of tracing obtained after such injections. It has been suggested that the fall following the primary rise, which is of short duration, is probably due to coronary artery contraction. This type of tracing seems to occur more frequently when extracts of the entire posterior lobe are used than when those of the pars intermedia alone are employed. It has been suggested that the variations from the common type which will be noted later may be due to the action of substances extracted from the nervous part of the posterior lobe.

Intravenous injection of 0.05 gm. of dried hypophyses from fetal pigs measuring 175 mm. extracted in 5 cc. of normal salt solution gave a decided pressor effect such as is usually obtained by the intravenous injection of extracts made from adult glands. The pressor reaction followed the injection rapidly and was long continued, being unassociated with any marked change in the tracing (Fig. 2).

Intravenous injections of saline extracts of hypophyses from fetal pigs measuring 125 mm. gave different results. In some cases a slight pressor effect following a primary fall was noted, while in other instances no change in the tracing was noted. Fig. 3 is a tracing obtained after intravenous injection of an extract of 0.08 gm. of dried hypophyses in 5 cc. of normal salt solution. The injection of this extract caused a fall in pressure which was followed by a rise slightly above the level existing before the injection was made. This reaction was of short duration, the pressure soon sinking to the level which existed before the injection was made. Another tracing was made after the injection of an extract of 0.06 gm. of dried hypophyses from pigs measuring 125 mm. extracted in 3 cc. of salt solution. The amount of extract injected was therefore practically the same as in the preceding experiment. The injection of this extract caused a fall in pressure followed by a rapid return to the

level existing before the injection was made (Fig. 4). An injection of the residue left after filtration of the preceding solution extracted in 5 cc. of normal salt solution caused another fall with a rapid return to the level existing before the injection. The fall in pressure observed in the last experiments occurs frequently after injections of glands, even after attempts to remove completely the depressor substance which exists in all parts of the gland have been made by thorough extraction with absolute alcohol.

Fig. 3 indicates that in some instances a slight but unmistakable pressor effect of short duration may follow the intravenous injection of extracts made from the hypophyses of pigs measuring 125 mm. This reaction does not follow the injection of all extracts made from the glands of pigs of this measurement. It is possible that in attempts to obtain fresh material a few pigs measuring slightly more than 125 mm. have been occasionally used, but if this is the case, they have exceeded this measurement only by a few mm.

I believe that I am justified in stating that the active principle of the pars intermedia, using the pressor substance as an index, begins to appear in fetal pigs measuring 125 mm. or slightly more.

It is somewhat difficult to determine the age of fetal pigs as estimated by measurements. Koch says that great difficulty is experienced in finding any statement concerning the age of pig fetuses. The statements of different authors do not always agree, but the two which come closest to an agreement are those of Bradley and Coe. Bradley compared the length of the embryos with the time of coition. Coe estimated the age from the rate of development of other mammals. While considerable uncertainty is attached to these figures, it may be assumed that the 50 mm. pig fetus is about 40 days old from conception; the 100 mm. fetus is 55 to 62 days old; and the 200 mm. pig is 88 to 90 days old from conception.

As determined by the pressor reaction the secretion of the pars intermedia seems to be established in pigs measuring 125 mm. The reaction is not obtained after the injection of all extracts of glands removed at this period and even when present is not marked. There are, however, often definite indications of a pressor effect as indicated in Fig. 3 As far as can be estimated, a pig measuring 125 mm. is about $9\frac{1}{2}$ or 10 weeks old from conception. During the period rep-

resented by the differences between 125 and 175 mm., the secretion of the pars intermedia becomes as active as that of the adult gland.

McCord[2] has recently attempted to determine the time of appearance of the active principle of the pars intermedia. Its presence was determined by the oxytocic activity of extracts of glands by means of the method of Dale and Laidlaw,[3] using histamine as a standard. The tests for pituitrin were begun with embryos at or near full term. These tests and others as far back as 9 weeks were quantitative tests. Pituitrin was found in the extracts of glands as early as 9 weeks, and the quantity for unit of weight was larger than in the adult. In the 7th and 8th weeks of fetal life the pituitary could no longer be recognized, although the sella turcica was plainly visible. The experiments, therefore, could not be made earlier than the 9th week of fetal life.

McCord worked with bovine fetuses and it is difficult to determine the relative ages of calf and pig fetuses by the lengths. McCord found that the first indication of an active pars intermedia occurs in a bovine fetus measuring 165 mm., while evidences of an active pars intermedia are found in a pig fetus measuring 125 mm.

Fenger[4] has shown that both the thyroid and suprarenal glands of the beef, hog, and sheep contain their active principles not only at birth, but also in the fetus. He believes that both the thyroid and suprarenals of the fetus take a distinct and active part in its growth.

SUMMARY.

The pressor substance of the hypophysis is so marked in the pig fetus measuring 175 mm. that it seems probable that a fetus of this length is independent of the secretion of the mother's hypophysis.

[2] McCord, C. P., The Occurrence of Pituitrin and Epinephrin in Fetal Pituitary and Suprarenal Glands, *Jour. Biol. Chem.*, 1915, xxiii, 435.

[3] Dale, H. H., and Laidlaw, P. P., A Method of Standardizing Pituitary (Infundibular) Extracts, *Jour. Pharm. and Exper. Therap.*, 1912–13, iv, 75.

[4] Fenger, F., On the Presence of Active Principles in the Thyroid and Suprarenal Glands Before and After Birth. Second Paper, *Jour. Biol. Chem.*, 1912, xii, 55.

EXPLANATION OF PLATES.

PLATE 96.

FIG. 1. Effect upon blood pressure of the intravenous injection of 0.1249 gm. of dried hypophyses removed from pigs just before birth, dissolved in 5 cc. of normal salt solution. This type of tracing is often produced by injection of extracts of the adult gland, but is not the most common form.

FIG. 2. Effect upon blood pressure of the intravenous injection of 0.05 gm. of dried hypophyses removed from pigs measuring 175 mm., dissolved in 5 cc. of normal salt solution. A prompt and long continued pressor effect is noted.

PLATE 97.

FIG. 3. Effect upon blood pressure of the intravenous injection of 0.08 gm. of dried hypophyses removed from pigs measuring 125 mm., dissolved in 5 cc. of normal salt solution. The fall in pressure is followed by a distinct but temporary rise.

FIG. 4. Effect upon blood pressure of the intravenous injection of 0.06 gm. of dried hypophyses removed from pigs measuring 125 mm., dissolved in 3 cc. of normal salt solution. A fall in pressure is noted with a rise to the level existing before the injection.

FIG. 1.

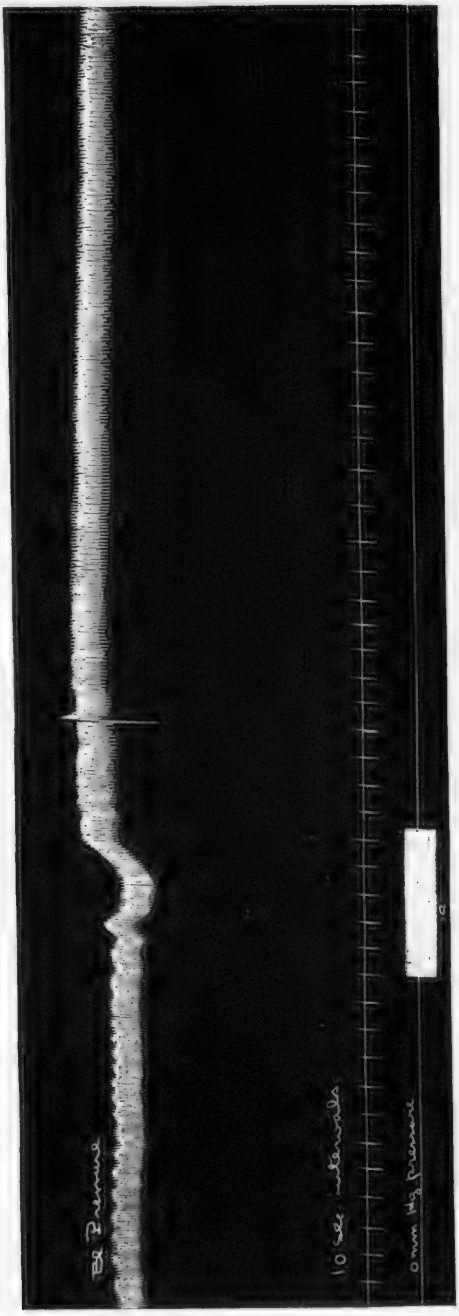

FIG. 2.

(Lewis: Pressor Substance in Fetal Hypophysis.)

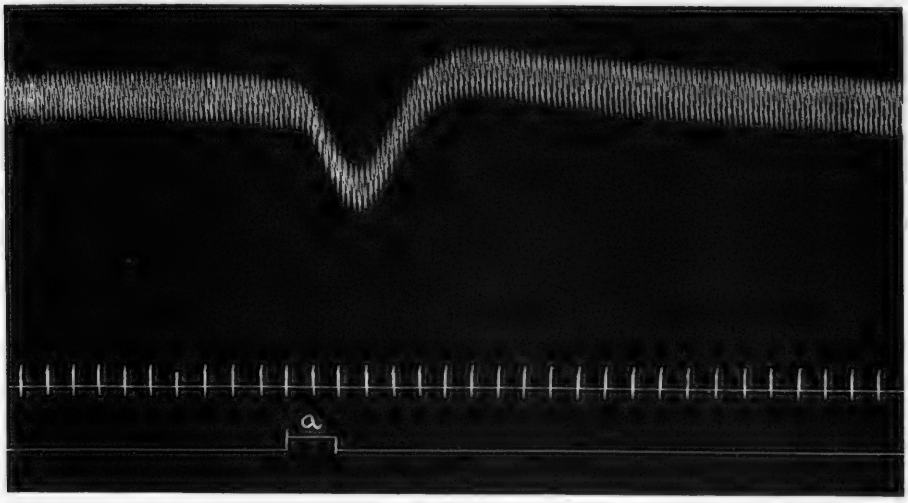

FIG. 3.

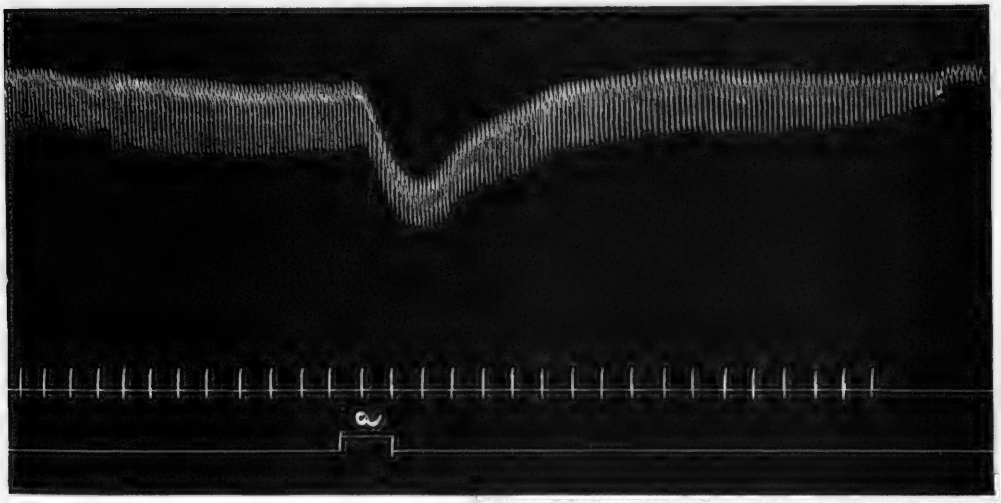

FIG. 4.

CHEMICAL VERSUS SERUM TREATMENT OF EPIDEMIC MENINGITIS.

By SIMON FLEXNER, M.D., and HAROLD L. AMOSS, M.D.
(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, March 20, 1916.)

INTRODUCTION.

The reappearance of epidemic meningitis among the belligerent armies in Europe has led to numerous reports on the treatment of the disease. For the most part, the antimeningococcic serum has been employed, but in one instance at least the attempt has been made to improve upon the serum treatment by substituting for it chemical treatment. This attempt to employ directly antiseptic drugs in the treatment of epidemic meningitis is a revival of an old notion.

The circumstances are such as to make the chemical treatment favorable from a theoretical standpoint. The infection tends to be local, that is, within the cerebrospinal membranes, and not general or throughout the body. The membranes are directly accessible from without, so that the chemical substance can be brought into immediate relation with the seat of infection. Moreover, the meningococcus as cultivated outside the body is not a highly resistant microorganism, but is readily injured by chemical action, and is indeed quickly injured by the waste products of its own growth. What, therefore, is apparently simpler or more promising than to control epidemic meningitis by the direct application by means of lumbar puncture of bactericidal chemicals to the infected and inflamed cerebrospinal membranes?

There is, moreover, still another theoretical reason for resorting to the chemical treatment of epidemic meningitis. The meningococcus is now known to be a microorganism which occurs in at least two varieties or types, distinguished as meningococcus and parameningococcus.[1] Their main differences are immunological, so that they

[1] Amoss, H. L., and Wollstein, M., *Jour. Exper. Med.*, 1916, xxiii, 403.

are subject to therapeutic influences through antisera only when correspondence exists between the infecting type of microorganism and the particular specific antibodies present in a serum. Presumably, chemical agents being less specific would be less restricted in their effects, and might be expected to act quite irrespective of the type of meningococcus causing the infection.

Wolff[2] has recently recommended the employment of protargol in the treatment of epidemic meningitis, basing the recommendation of its use on eight cases, of which five recovered. The point of departure in the selection and administration of protargol was the successful employment of so called colloidal silver preparations in the treatment of gonorrhea, and the close relationships as regards conditions of culture, survival, and reaction to antiseptics subsisting between gonococci and meningococci. It is conceded by Wolff that because of the highly variable clinical course of epidemic meningitis, the number of cases which he reports is too small to permit of the drawing of any conclusions regarding the therapeutic efficiency of the chemical. He believes, however, that it is at least innocuous and can, therefore, be administered with the assurance that if it is not curative, it will do no harm.

The first method of direct chemical treatment of epidemic meningitis to receive considerable attention was that in which lysol was employed. The method was introduced in Lisbon during the epidemic which prevailed there in the first years of the present century, and was briefly reported on by Seager[3] in 1902. Following Seager's report, lysol was tested in the course of the epidemic from which Greater New York was suffering at the time.[4] This epidemic continued for several years and led to the working out of the serum treatment, but at that early date no specific means of combating the disease was known.

It is remarkable and instructive to consider that in these instances, namely, the one relating to lysol and the other to protargol, what appeared to be preliminary success was achieved by the chemical treatment. Wider experience, however, led to the abandonment of

[2] Wolff, G., *Deutsch. med. Wchnschr.*, 1915, xli, 1486.
[3] Seager, H. W., *Lancet*, 1902, ii, 1188.
[4] Manges, M., *Med. News*, 1904, lxxxiv, 913.

the lysol treatment. In the meantime, knowledge has been gained and methods perfected through which the value of a proposed form of treatment of epidemic meningitis can be determined experimentally in animals, with a degree of accuracy exceeding that based on clinical and limited statistical observations on human beings.

It happens that epidemic meningitis in man is a highly variable disease. The severity fluctuates between clinical conditions so slight as to justify the term abortive being applied to certain cases, and at other times so severe (fulminant) as to cause death swiftly, and sometimes with the first appearance of symptoms. Hence the mortality percentage of the disease varies in different epidemics and places. To determine approximately what the effects of varying modes of treatment are it is necessary to compare coincidently treated and untreated cases of sufficient number. A definite result is therefore obtained only after some time, and by comparing figures obtained in different localities and over a comparatively long period of time. So long as the figures based on small local observations alone are available, no final deduction can be made.

The employment of animals for the test is free of the uncertainty of the statistical method. The dosage of the culture of meningococcus can be adjusted to produce the result desired. Then the effect and the manner of the therapeutic action can be accurately ascertained. Tests of this kind have been made with protargol and for comparison with lysol and with serum, with results unfavorable to the chemical treatment.

EXPERIMENTAL.

The experimental studies on epidemic meningitis carried out during the past 10 years have shown two kinds of animals to be suitable for determining the pathogenic action of meningococci and the effects of therapeutic agents in meningococcic infection. The animals are monkeys[5] and young guinea pigs.[6] While the age of the monkeys seems not to matter, older guinea pigs are highly resistant to meningococcic infection. The cultures of meningococci are injected into

[5] Flexner, S., *Jour. Exper. Med.*, 1907, ix, 142.

[6] Flexner, S., *Jour. Exper. Med.*, 1907, ix, 168.

the peritoneal cavity in the guinea pigs and into the subarachnoid space in the monkeys, in the latter by means of lumbar puncture. The injected meningococci set up local inflammations in the exudate attending which the fate of the microorganism can be followed precisely, as in human cases of epidemic meningitis in which the cerebrospinal fluid is removed from time to time by lumbar puncture.

The changes occurring in the meninges or the peritoneum vary with the activity and the dose of the culture. Non-virulent cultures are quickly phagocyted, no considerable multiplication takes place, and the inoculated animals show few or no symptoms and recover fully in 24 hours. Active cultures multiply, are imperfectly phagocyted, and cause death according to the virulence and dose in 6 to 8 to 12 hours in guinea pigs, and in 20 to 48 hours in monkeys. At autopsy living meningococci are present in the peritoneal or meningeal exudate and in the blood. Film preparations show meningococci free and phagocyted also in the peritoneal fluid and more numerously in the omentum of the guinea pig, and both free and phagocyted in the exudate in the pia-arachnoid membranes of the monkey.

In carrying out the experiments a virulent strain of the meningococcus (Isadore) was employed. Tests made to determine the optimum period of growth at which to employ the culture indicated it to be after about 18 hours' incubation at 37°C. Sheep serum dextrose agar was used as a medium. Growths 24 hours old infected far more irregularly than those 18 hours old. The reason for the disparity is found in the spontaneous degeneration of the culture, which probably has already begun at 18 hours, and can be detected by microscopic examination at 22 hours. In the guinea pigs it was found in this instance, as already described,[1] that consistent results are best attained by employing one minimal lethal dose of the culture. In this way, lack of protective power is made to assert itself readily, while the existence of protective power is easily demonstrated and can be further investigated. In monkeys the infecting dose is easily determined, although the fatal dose is established less readily. While these animals were used sparingly, the protocol in each instance indicates the outcome of the therapeutic test compared with the control. Besides the result based on death or survival, the changes

taking place in the cerebrospinal fluid as followed by lumbar puncture, and the clinical course of the experimental disease, to a less definite degree, are also valuable indications of the action of the therapeutic agent.

Tests with Guinea Pigs.

The guinea pigs ranged in weight from 90 to 110 gm. The injections were intraperitoneal. The protargol was employed in 0.2 per cent strength, prepared by suspending the dried powder in sterile distilled water. The cultures of meningococci on sheep serum agar after 18 hours' incubation were suspended in sterile salt solution, and injected either immediately or after such intervals as are indicated in the separate experiments. The dose was one m.l.d.

TABLE I.

Experiment 1. Toxicity of Protargol.

Weight of guinea pig.	Quantity of suspension injected.	Result.
gm.		
90	0.3 cc. + 0.7 cc. water.	Survived.
90	0.5 " + 0.5 " "	"
90	1.0 " + 1.0 " "	"
90	2.0 " + 2.0 " "	Died in 38 hrs.

The tests shown in Table I may be taken to indicate that the toxic dose of protargol for young guinea pigs is well above the dose employed for therapeutic purposes.

Experiment 2. Therapeutic Tests of Protargol Intraperitoneally in the Guinea Pig.—One m.l.d. of living culture in a total volume of 1 cc. was injected intraperitoneally into young guinea pigs. The amount of 0.2 per cent protargol suspension given was made up to 1 cc. with sterile water and injected at the time indicated in Table II.

TABLE II.

Weight of guinea pig.	Dose of protargol suspension.	Time of administration.	Result.
gm.	*cc.*		
110	0.5	Immediately.	Death in 12 hrs.
110	0.5	"	" " 21 "
95	0.5	"	" " 15 "
100	0.5	"	" " 10 "
99	0.1	"	" " 8 "
82	0.1	"	" " 19 "
82	0.1	"	" " 11 "
100	0.5	After 15 min.	" " 12 "
110	0.7	" 15 "	" " 8 "
100	0.5	" 1 hr.	" " 10 "
75	0.1	" 1 "	" " 15 "
75	0.1	" 1 "	" " 18 "
95	0.25	Mixed with culture, injected after 1 hr.'s contact.	" " 25 "
110	1.00	" " "	" " 14 "

This experiment is conclusive in demonstrating that protargol in 0.2 per cent suspension, the strength employed by Wolff,[2] is incapable of preventing in guinea pigs the lethal effect of a single minimal fatal dose of the meningococcus, whether administered combined with the culture immediately after the mixture, or whether the culture and protargol are injected separately 15 minutes apart. The period of survival of the treated animals may be about the same as or somewhat greater than the controls. Living meningococci are always found in the peritoneal cavity and in the heart's blood. In this connection it is interesting to find that when the culture and protargol suspension which have been in contact for 1 hour are transplanted to fresh sheep serum agar, no growth is obtained; while, however, cultures from the peritoneal cavity of the dead animals which were inoculated with the mixture are positive, thus indicating that the protargol merely inhibits the growth of the meningococci, but may not destroy them in 1 hour's time. This observation raises the question whether the failure of the protargol is due merely to its imperfect bactericidal power, or whether it depends upon lack of antitoxic power at the same time. The next experiment bears on this consideration.

Experiment 3. Therapeutic Test of Protargol with Autolysate.—An autolysate[7] of the meningococcus was prepared in the usual way by suspending the culture in salt solution, adding toluene, incubating, allowing the toluene to evaporate, and centrifugalizing. The clear supernatant fluid was employed. The autolysate and protargol suspensions were mixed and immediately injected, as shown in Table III.

TABLE III.

Weight of guinea pig.	Dose of autolysate.	Dose of suspension of 0.2% protargol.	Result.
gm.			
90	0.25 cc. in 1 cc. salt solution.	None.	Died in 7 hrs.
100	0.25 " " 1 " " "	0.5 cc.	" during night.
110	0.25 " " 1 " " "	1.0 "	" " "

This experiment indicates that protargol does not exhibit pronounced antitoxic properties, although a delay was noted in the fatal issue in the animals in which it was given.

Mechanism of the Action of Protargol and of Antiserum.

In carrying out the above tests which were made on several different occasions, control observations with antimeningococcic horse serum were also made. The quantity of serum administered was 0.5 cc., and in every one of the six instances in which the antiserum was given the animal survived. The antiserum was also injected in association with the protargol, without, however, preventing a fatal issue. On the other hand, the antiserum influences directly the mechanism of action of protargol, although unable apparently to overcome wholly some injurious effect which it exerts. The next experiment, therefore, was devised to bring out definitely the mechanism of action of the protargol in comparison with the manner of action of the antiserum.

Experiment 4. Mechanism of the Action of Protargol.—Four guinea pigs, ranging in weight from 90 to 110 gm., were employed. Besides the inoculation of meningococci, one of them received immediately 0.5 cc. of a suspension of 0.2 per cent protargol alone; the second received 0.5 cc. of antiserum alone; and

[7] Flexner, S., *Jour. Exper. Med.*, 1907, ix, 105.

the third 0.5 cc. of a suspension of protargol and 0.5 cc. of antiserum in immediate succession. The fourth animal received only the culture. All were chloroformed at the expiration of 6 hours, at which time the control animal was prostrate and the others were definitely ill.

The autopsies were made at once and the distribution of meningococci in the abdominal cavity studied by means of cultures and film preparations, and in the blood of the heart by cultures alone.

Guinea Pig 1. Protargol Alone.—Two drops of the blood of the heart were laked in 0.5 cc. of sterile water and plated in sheep serum agar. The colonies numbered about 2,000. Two drops of the slightly turbid peritoneal fluid were similarly plated. The colonies numbered about 2,000. The film preparations from the fluid in the abdominal cavity showed numerous scattered meningococci and a notable absence of polynuclear cells, and the omentum showed scattered free meningococci and polynuclear leukocytes, of which a very small number contained diplococci, the leukocytes themselves staining feebly and appearing degenerated.

Guinea Pig 2. Antiserum Alone.—Two drops of the heart's blood were treated as in No. 1. No colonies. Two drops of the somewhat turbid peritoneal fluid gave about 2,500 colonies. Film preparations of the peritoneal fluid showed numerous well preserved polynuclear leukocytes containing many meningococci, while the extracellular diplococci, small in number, were arranged in small clumps. The film preparations from the omentum exhibited many more leukocytes, most of which were filled with meningoccoci in various stages of disintegration, the leukocytes themselves appearing in normal condition. A few extracellular agglutinated meningococci were also present.

Guinea Pig 3. Protargol and Antiserum.—Two drops of the heart's blood yielded one colony, and two drops of the clear peritoneal fluid about 150 colonies. Film preparations of the peritoneal fluid showed a smaller number of diplococci than in either of the preceding animals. Polynuclear leukocytes were present, but in far smaller number than in No. 2, and many were degenerated and had rarely phagocyted the diplococci. Such meningococci as were present at all were agglutinated. The films from the omentum showed somewhat larger numbers of degenerated polynuclear cells, of which a part contained meningococci, and quite numerous free, single and clumped diplococci.

Guinea Pig 4. Control.—Two drops of the heart's blood gave about 2,000 colonies, and two drops of the clear peritoneal fluid innumerable colonies. Congestion of the peritoneal organs was more marked in this animal than in the others. Film preparations of the peritoneal fluid showed a very large number of particularly well preserved meningococci, but no polynuclear leukocytes, and those of the omentum showed a similar condition. The absence of polynuclear cells from the latter structure is particularly noteworthy.

This experiment confirms the observation made upon the guinea pigs treated with protargol which were allowed to succumb. Its

significance is clear. Protargol has some power to cause dissolution of the meningococci with which it comes in contact, but does not destroy all. A later increase occurs and invasion of the blood stream takes place. Probably the injurious action of the protargol arises from its antileukotactic properties, for it causes degeneration of the polynuclear leukocytes and prevents phagocytosis, both *in vivo* and *in vitro*.

This particular action of the protargol is rendered obvious at once on comparison with the manner in which the antiserum acts. In this instance there is a diversion of polynuclear leukocytes in large numbers to the peritoneal cavity and an acceleration of phagocytosis. Hence at a period at which many living meningococci are still present in the abdominal cavity, practically no escape is taking place into the general blood stream. The addition of the antiserum to the protargol obviates partially the antileukotactic effect of the latter, but does not completely overcome it, because of the injury inflicted by the chemical on the leukocytes brought out by the antiserum.

Protargol is, therefore, bactericidal for the meningococcus, as for the gonococcus, through direct chemical action, but it is not curative in meningococcic infection in the guinea pig, because being confined within the abdominal cavity it exerts a passive chemotactic and antiphagocytic influence on the polynuclear cells which are themselves injured and destroyed through its action. Thus the imperfect bactericidal effect is inadequate to control infection, while the antileukotactic action is itself harmful and promoting of infection. Moreover, the masses of protargol are themselves taken up by leukocytes, to be transported doubtless to organs in which the substance may remain indefinitely.

Tests with Monkeys.

The inoculation of virulent meningococci into the subarachnoid space in monkeys causes an acute inflammation which extends throughout the meninges and into the cerebral ventricles. Depending upon the pathogenic power and the dose, the animal which develops symptoms within a few hours may die within 24 hours or may recover. According as one or the other result is obtained, the meningococci multiply and are imperfectly phagocyted, or they increase little

and are perfectly phagocyted. The cerebrospinal fluid at first remains clear, then becomes turbid with emigrated polynuclear leukocytes; finally it may again become clear. A blood invasion often follows within a few hours of the inoculation, but is rarely present at the end, even in fatal cases.

Experiment 5. Control.—A *Macacus rhesus* was injected at 11 a.m. intraspinally with 2 cc. of a salt solution suspension, representing 1½ agar slant cultures of meningococcus, the strain being the same as that used in the experiments on the guinea pigs. 5.00 p.m., animal sick. Lumbar puncture yielded a fluid already turbid, giving positive culture and showing on films many meningococci, few leukocytes, and little phagocytosis. Blood taken from arm vein gave positive cultures. 9.00 a.m. the next day, prostrate. Lumbar puncture yielded highly turbid fluid, giving positive cultures and films in which leukocytes were numerous and of which many contained meningococci; free diplococci were also present. Death at 3.30 p.m. Survived 20½ hours.

Autopsy.—The membranes of the brain and spinal cord showed turbid fluid and congestion. The cultures and film preparations confirmed previous findings. Both intra- and extracellular meningococci were present. Cultures positive. Cultures of heart's blood negative.

Sections from the brain and spinal cord show an acute fibrinopurulent meningitis, most pronounced over the convex surface of the brain. The ventricles are involved slightly. Many diplococci are present in the exudate and are contained chiefly, if not wholly within leukocytes.

Experiment 6. Treatment with Antimeningococcic Horse Serum.—8.30 a.m., a *Macacus rhesus* received intraspinally an injection of a suspension of meningococci as in Experiment 5. 12.30 p.m., lumbar puncture yielded cloudy fluid, giving positive cultures. Films showed numerous meningococci, pus cells, and phagocytosis already present. The culture from the heart's blood was negative. 2 cc. of antiserum were injected. 4.30 p.m., animal appeared well; lumbar puncture yielded turbid fluid, giving positive culture and showing in the film marked phagocytosis and agglutination of all extracellular meningococci. 8.30 p.m., active. 28 hours after the inoculation the animal appeared well; lumbar puncture yielded a turbid fluid which gave a negative culture. The films showed many leukocytes, some containing meningococci and very few extracellular diplococci. A number of endothelial cells and lymphocytes were present. The animal remained well.

Experiment 7. Treatment with Protargol.—8.30 a m., a *Macacus rhesus* received an equal part of a suspension of the culture used in the previous experiments. 12.30 p.m., little change in condition. Lumbar puncture yielded a cloudy fluid, from which cultures were positive and films show many leukocytes and well advanced phagocytosis of meningococci; some free meningococci were also present. Injected 2 cc. of 0.2 per cent protargol suspension. 2.30 p.m., animal

lying down, drowsy, surface cold, limbs spastic. 4.00 p.m., respiration rapid, slight convulsion recurring frequently. Blood withdrawn at this time gave a positive culture. 4.30 p.m., died. Survived 8 hours.

Autopsy.—No lesions were found outside the cerebrospinal membranes. The meninges contained an excess of almost clear fluid. No marked congestion. Cultures from the meninges of the brain and spinal cord yielded luxuriant growth. Films showed a large number of meningococci, both intra- and extracellular, and a widespread degeneration and even disintegration of leukocytes. The meningococci stain sharply and deeply. The choroid plexus of the lateral ventricle and the meninges of the olfactory lobes gave results similar to those of the other membranes. Cultures from the heart's blood were positive. Moreover, cultures prepared from the nasal mucosa yielded, among other bacteria, typical meningococci, possessing identical agglutination reactions with the strain employed for inoculation.

The microscopic examination of sections from the central nervous organs shows many pus cells, with here and there red corpuscles, and no fibrin. The number of meningococci present is very large. Some degree of phagocytosis exists; but a striking phenomenon is the packing of lymph spaces with innumerable diplococci. They invade the pial spaces, infiltrate the superficial layers of the cerebral cortex, and follow the perivascular spaces for some distance into the cortex. Certain coagula of red and white corpuscles within the central ventricles also enclose diplococci.

A comparison of the results in the three monkeys inoculated with meningococci and left in one case untreated, and in the others treated either with antiserum or protargol, is unfavorable to the treatment with protargol. The untreated or control animal succumbed under conditions indicating definite if inadequate reaction against and resistance to the infection. The serum-treated animal was already ill when the antiserum was administered, the effect of which was to disperse almost immediately all the symptoms and lead to prompt recovery, coincidently with the removal by phagocytosis of the meningococci. The third monkey exhibited practically no symptoms 4 hours after the inoculation when the protargol was given, but prostration and rapid intensification of symptoms followed almost immediately. Death occurred 4 hours later. The causes of the unfavorable results are to be sought in the two or three main effects of the protargol: (*a*) on the leukocytes, which already present, were greatly injured; (*b*) on the meningococci, which doubtless were also affected through direct bactericidal action from which toxic substances were set free; and (*c*) finally on removal of all restraint to

multiplication of surviving diplococci, since the immense numbers present in the lymph spaces as well as in the exudate indicate rapid growth.

A repetition of the experiment in which protargol was used was made in Experiment 8.

Experiment 8. Treatment with Protargol.—11.15 a m., a *Macacus rhesus* received intraspinal inoculation of one agar slant culture, 18 hours old. 3.15 p.m., slightly ill; on perch. Lumbar puncture yielded cloudy fluid; culture positive. Films showed few leukocytes and many free meningococci. Injected 2 cc. of 0.2 per cent protargol suspension. 4.00 p.m., animal prostrate and spastic. 5.00 p.m., refused to rise. Died 5.00 a.m. next day. Survived 18 hours.

Autopsy.—No obvious visceral lesions outside the central nervous system. Turbid fluid in meninges, especially evident in sulci of cerebral convolutions. The general gray color of the cortex was about normal, but two symmetrical rectangular areas measuring 2 by 4 cm. were deeply congested and contained petechial hemorrhagic spots. They lay anterior and posterior to the fissure of Sylvius and extended to the midparietal sulcus comprising the ascending and inferior marginal convolutions. A few separate hemorrhages existed in the first temporal lobe, and the congestion and hemorrhages appeared to be confined to the gray matter. Cultures from the heart's blood and from several levels of the spinal cord and different surfaces of the brain were positive for the meningococcus. Film preparations showed many diplococci, few if any leukocytes, and little phagocytosis. Impression films prepared from the congested area of the brain contain scattered meningococci, few leukocytes, and almost no phagocytosis.

Sections from the brain show edema of and many leukocytes and red corpuscles within the meninges. The axudate contains many diplococci, both extra- and intracellular. In places the diplococci extend into the superficial layer of the cortex and slightly into the perivascular lymph spaces. These are far less numerous than in the 8 hour specimens. The affected cortex is congested and is the seat of many hemorrhages of varying size. The escape of blood is partly into the tissues and partly into the perivascular lymphatics. There is no obvious special relation between the hemorrhages and the diplococci.

A control for this monkey which received an identical quantity of an 18 hour culture was made ill, but recovered without treatment and following the spontaneous phagocytosis of the meningococci.

The differences in general disadvantageous to the protargol arose also in instances in which neither the untreated control nor the treated monkeys succumbed. In these instances cultures 24 hours old were employed. The symptoms set in more slowly than when 18 hour cultures were used and became less severe, abating wholly later.

But the monkeys given protargol suffered intensification of the symptoms almost immediately after the injection and recovered much more slowly than the untreated animals. In conformity with this fact it was found that the meningococci survived longer in the cerebrospinal fluid in the treated animals than in the control. A single experiment is given to bring out the distinction mentioned.

Experiment 9. Control.—8.50 a.m., a *Macacus rhesus* received an intraspinal inoculation of 1¾ agar slant cultures of meningococci. 2.50 p.m., slightly ill. Turbid lumbar puncture fluid gave growth. The films showed numerous leukocytes, few meningococci, and little phagocytosis. Blood cultures were negative. The symptoms progressed slowly and never became severe. 24 hours after inoculation, the animal was still ill, but improving. Lumbar puncture yielded turbid fluid, which gave negative cultures. Film preparation of the fluid showed numerous leukocytes and few meningococci all within phagocytes. Within another 24 hours the animal had recovered.

Experiment 10. Treated.—8.30 a.m., a *Macacus rhesus* received intraspinally 1½ agar slant cultures. 12.30 p.m., ill. The turbid lumbar puncture fluid gave positive cultures. The film showed many leukocytes, few meningococci, and slight phagocytosis. Injected 1 cc. of 0.2 per cent protargol suspension. 1.30 p.m., very ill. 5.00 p.m., condition unchanged; blood culture positive. Turbid lumbar puncture fluid gave positive cultures. Film showed many leukocytes, of which some contained meningococci; many extracellular meningococci also. 10.00 p.m., animal lying on bottom of cage; aroused with difficulty. 24 hours after inoculation, improving. Turbid lumbar puncture of fluid still showed intra- and extracellular diplococci. No growth was obtained in culture. At the expiration of another 24 hours, the animal had still further improved, but several days elapsed before recovery was complete.

Effects of Lysol.

The fact has already been mentioned that lysol has been employed in the treatment of epidemic meningitis with at first, as was supposed, beneficial results. Although a wider employment led very soon to its abandonment, yet the outcome of the studies of protargol suggested determining also the manner in which lysol acts. For this purpose small guinea pigs were employed. Two sets of tests were made: one in which the inoculated animals were allowed to succumb, the other in which they were chloroformed at the expiration of 6 hours, in order to ascertain just what changes were taking place in the peritoneal cavity. The lysol was employed in

1 per cent solution, which was the strength used in human cases of meningitis. The injection was made immediately following the injection of the culture, both being given into the peritoneal cavity (Table IV).

TABLE IV.

Experiment 11. Therapeutic Test of Lysol.

Weight of guinea pig.	Quantity of culture inoculated.	Treatment.	Result.
gm.			
100	1 m.l.d.	None (control).	Died in 10¾ hrs.
100	1 "	1 cc. lysol (control).	Survived.
100	1 "	0.5 cc. lysol.	Died in 7 hrs.
100	1 "	0.5 cc. lysol + 0.5 cc. antiserum.	" "32 "
100	1 "	0.25 cc. lysol + 0.5 cc. antiserum.	" "20 "
100	½ "	0.5 cc. lysol.	" "12 "

Guinea Pig 1. Control.—Peritoneal fluid clear. 0.05 cc. plated gave innumerable colonies of meningococci, as did the same quantity of heart's blood. Films of peritoneal fluid showed enormous numbers of meningococci, but no cells. Films from the omentum showed extremely few leukocytes containing meningococci.

Guinea Pig 2.—Considerable amount of clear peritoneal fluid. 0.05 cc. plated gave innumerable colonies. Same amount from heart's blood gave 250 colonies. Films of the peritoneal fluid showed large numbers of diplococci, well preserved but agglutinated into small clumps. No leukocytes present. Films of the omentum showed a considerable number of leukocytes, many containing diplococci and many extracellular diplococci agglutinated.

Guinea Pig 3.—Very small amount of turbid peritoneal fluid. 0.05 cc. plated gave innumerable colonies. Same quantity of heart's blood gave 200 colonies of meningococci. Films of peritoneal fluid showed numerous leukocytes filled with diplococci, and large numbers of diplococci arranged in small clumps remaining extracellular.

Guinea Pig 4.—Small amount of turbid peritoneal fluid. 0.05 cc. plated gave 1,000 colonies. Same quantity of heart's blood gave no colonies. Films of peritoneal fluid showed numerous leukocytes, some staining badly, many containing diplococci, and a considerable number of small clumps of extracellular diplococci, some partly disintegrated. Films of the omentum showed more phagocytosis and a smaller number of extracellular diplococci.

Guinea Pig 5.—A considerable amount of turbid peritoneal fluid. 0.05 cc. plated gave innumerable colonies, as did the heart's blood. Films of the peri-

toneal fluid showed many leukocytes and a small amount of phagocytosis, besides extracellular clumps of diplococci. Films of the omentum showed more phagocytosis than the peritoneal fluid and a larger number of leukocytes, besides extracellular diplococci.

Guinea Pig 6.—A considerable amount of turbid peritoneal fluid. 0.05 cc. plated gave innumerable colonies, as did the heart's blood. Films of the peritoneal fluid showed numerous leukocytes, a small amount of phagocytosis, and numerous clumped extracellular diplococci. Films of the omentum showed numerous leukocytes, slight phagocytosis, and extracellular diplococci occurring in clumps.

Mechanism of the Action of Lysol.

The mode of the action of lysol was studied by inoculating three young guinea pigs with one minimal lethal dose of culture and treating them immediately with 1 per cent lysol or 1 per cent lysol plus antiserum, as indicated in Table V.

TABLE V.

Experiment 12.

Weight of guinea pig.	Quantity of culture inoculated.	Treatment immediately after inoculation.	Condition after 6 hrs.
gm.			
100	1 m.l.d.	0.5 cc. lysol + 0.5 cc. water.	Dead.
100	1 "	0.25 " " + 0.75 " "	Very sick.
100	1 "	0.25 " " + 0.25 " " + 0.5 cc. antiserum.	Prostrate.

The two guinea pigs which still survived were chloroformed and autopsies performed at once.

Guinea Pig 1.—Peritoneal fluid clear. 0.05 cc. plated gave innumerable colonies of meningococci. Same quantity of heart's blood gave 100 colonies. Films of the peritoneal fluid showed many meningococci and few leukocytes, there being almost no phagocytosis, while the leukocytes stained feebly. The meningococci were chiefly aggregated into clumps of 10 or 20 individuals. They stained as a rule diffusely and not sharply. The omentum showed many more leukocytes, almost all containing meningococci singly and in small clumps. The phagocytes stained feebly.

Guinea Pig 2.—Peritoneal fluid clear. 0.05 cc. gave innumerable colonies of meningococci. Same quantity of heart's blood gave about 100 colonies. Films of peritoneal fluid and omentum showed a condition similar to No. 1.

Guinea Pig 3.—Peritoneal fluid turbid, gave 150 colonies of meningococci and the same quantity of heart's blood gave three colonies. Films from the peritoneal cavity showed no diplococci whatever, either free or in leukocytes, of which a good number in excellent preservation were present. Films from the omentum showed large numbers of well preserved leukocytes, of which a few contained meningococci, either sharply stained or disintegrating; extracellular diplococci were practically absent.

It may in brief be stated that the experiments with guinea pigs indicate that lysol is antileukotactic to an even greater degree than is protargol. Moreover, the antiserum is capable in this case of overcoming to even a further extent the negative chemotactic and antiphagocytic action of the chemical. Obviously the combined toxic power of the chemical and the disintegrating effect upon the meningococci, notwithstanding the action of the serum, bring about a fatal issue.

DISCUSSION.

The results of the tests with protargol and with lysol are consistent. The chemicals have shown themselves not to be curative, but rather to be injurious in experimental meningococcic infection in guinea pigs and monkeys. Far from exhibiting power to convert a fatal into a non-fatal issue, they have shown rather a reverse tendency. Even when, as in certain tests on monkeys, recovery occurred after employing protargol, the symptoms of the treated animals were intensified as compared with the untreated control animals.

The explanation of the unfavorable effect of the chemicals is supplied by the study of the processes occurring in the peritoneum of guinea pigs and the subarachnoid space of monkeys. The key to recovery from infection with the meningococcus is furnished by the phenomenon of phagocytosis. Whatever means promote phagocytosis under conditions in which the leukocytes remain potent, facilitate recovery; whatever means lead to the reverse effect interfere with recovery. The chemicals have been shown to be antileukotactic, antiphagocytic, and indeed to be cell poisons of considerable power. Hence when they are brought into relation with the seat of infection, they prevent emigration of leukocytes on an adequate scale, and they reduce phagocytosis by such leukocytes as have entered the serous cavities.

Moreover, they also injure and bring about degeneration of the leukocytes themselves.

The resultant of this set of injurious activities is to open the way for a free invasion of the blood by the meningococci and for almost unrestrained multiplication in the serous cavities.

The chemicals do exert a direct bactericidal action upon such meningococci as come under their influence under conditions of suitable concentration, but not all the meningococci are thus destroyed. Those which survive multiply almost without hindrance; while the absence of detoxicating power on the part of the chemical permits the disintegration products of the destroyed meningococci to exert their poisonous effect upon the animal organism.

The manner of action of the chemicals is precisely the reverse of the antiserum. The latter acts leukotactically and brings into the infected serous cavities a far larger number of leukocytes than would otherwise appear there in the same period of time. Moreover, by preparing the meningococci for phagocytosis by supplying opsonin and by facilitating that process by agglutinating the diplococci, the serum greatly promotes the englobing of the microorganisms. Finally, through the possession of antitoxic power it neutralizes whatever endotoxin may be liberated by the disintegrating diplococci.

It is, therefore, not remarkable to find, as the experiments have shown, that the antiserum is capable of overcoming part of the defects of the chemicals. Because of its leukotactic and phagocytic properties, the antiserum removes some of the damage which the chemicals produce on account of their antagonism to those essential phenomena. But combinations of the chemical and the antiserum have proven less effective in combating experimental meningococcic infection than the antiserum alone, from which the deduction may be made that whatever benefit may attach to effective direct bactericidal action by the chemicals, irrespective of the type of meningococcus causing the infection, is lost because of the dangers arising from their antiphagocytic effects and lack of detoxicating properties.

Doubtless the introduction of the chemicals into the subarachnoid space in man has been made without the uniformly serious consequences observed in our experiments on animals. The reason for this

discrepancy is perhaps obvious. The relative dosage, considering comparative size and weight, was far less in man than in the animals. Hence it may be assumed that the injurious effects, if any were produced, were masked. To assume, on the other hand, that because no evidence of untoward action was detected in the treatment with protargol, and five out of eight cases of epidemic meningitis treated with that chemical recovered, the drug has curative properties, is to disregard previous experience with simple lumbar puncture, with lysol, and with other methods of treatment which for a time and because of the variable severity of cases of epidemic meningitis seemed to offer encouragement. It is not probable that such active protoplasmic poisons as protargol and lysol can be employed with impunity for direct introduction into the closed cavity of serous membranes, the seat of the meningococcic infection.

SUMMARY.

Claims of efficiency have been made at two widely separated periods for the chemical treatment of epidemic meningitis, in the first instance for lysol and in the second for protargol. The use of lysol was long since abandoned; the recommendation for protargol is based on a single series of cases, small in number. Because of the variable severity of epidemics of meningitis, small reliance can be placed on results of treatment limited in extent to small numbers of cases and to one locality. A more uniform and accurate measure of the value of a method of treatment is provided by animals infected experimentally with pathogenic cultures of meningococci.

Young guinea pigs respond in a definite manner to intraperitoneal inoculation of virulent meningococci. Neither protargol nor lysol proved to have any curative action on the experimental infection thus produced in these animals.

Monkeys respond in a characteristic manner to the inoculation of virulent cultures into the subarachnoid space. Protargol displayed no curative action on the experimental infection thus produced in these animals.

On the contrary, both lysol and protargol exert antileukotactic and antiphagocytic effects, and are also potent protoplasmic poisons,

and the leukocytes with which they come in contact are injured and made to degenerate. According to the extent to which these harmful properties are exerted, the chemicals promote the advance rather than restrain the progress of meningococcic infection.

Recovery from meningococcic infection in man and animals is accomplished chiefly through the process of phagocytosis. The specific antiserum acts curatively by increasing the emigration of leukocytes, by promoting phagocytosis directly, and by agglutinating the meningococci, and also by neutralizing endotoxin. Any means which interfere with and reduce these essential processes retard or prevent recovery. Both lysol and protargol interfere with and diminish the emigration of leukocytes and the phagocytosis of meningococci, and neither possesses antitoxic power.

The mixture of antiserum with lysol and with protargol reduces to a certain extent the antileukotactic and antiphagocytic effect of the chemicals; but this action is insufficient to set aside wholly the injurious effects which they produce.

It follows, therefore, that whatever theoretical advantages might accrue from a bactericidal activity exerted by these chemicals independently of the type of meningococcus causing epidemic meningitis, is more than offset by the harmful effects which they cause.

Hence specific antiserum seems to provide the logical therapeutic agent with which to combat epidemic meningitis, since it is itself innocuous and promotes those processes essential to recovery from the disease. The problem up to the present has been that of producing an antiserum which represents the several types of the meningococcus, and this problem is now in a fair way to being solved.[1]